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Telomere Length in Women with Breast Cancer: A Longitudinal Study of their Relationship to Chemotherapy and Acquired Psychoneurological Symptoms

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Telomere Length in Women with Breast Cancer: A Longitudinal Study of their Relationship to Chemotherapy and Acquired Psychoneurological Symptoms

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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To My Parents

Table of Contents

List of Tables

List of Figures

Abstract

TELOMERE LENGTH IN WOMEN WITH BREAST CANCER: A LONGITUDINAL STUDY OF THEIR RELATIONSHIP TO CHEMOTHERAPY AND ACQUIRED PSYCHONEUROLOGICAL SYMPTOMS

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Breast Cancer (BC) is one of the most commonly diagnosed malignancies in females. The 5-year survival rate for women with early stage BC is about 90%. However, the quality of life (QOL) for many of these women may be adversely affected due to treatment/cancer–related side effects, including a constellation of symptoms (anxiety, depression, pain, fatigue, sleep disturbance and depression), that are collectively termed psychoneurological symptoms (PNS). To gain insight into the contributory role of telomere length (TL) in the development and persistence of PNS, we have longitudinally studied 72 women (ages 23-71) with early stage BC (I-IIIA) at 5 time points: prior to chemotherapy (baseline), prior to the fourth cycle of chemotherapy (mid-chemo), 6

months, 1 year and 2 years following the initiation of chemotherapy. Measures quantified included TL [using both a monochrome multiplex qPCR assay (at 5 time points) and a semi-quantitative chromosome-specific fluorescence in situ hybridization (FISH) assay (at baseline and mid-chemo)] and scores for each PNS. Variables predictive of qPCR mean TLs were age (p=0.004) and race (African Americans had greater mean TLs than Caucasians; p=0.019). While visit was not found to be a significant predictor of mean TLs (p=0.666), chromosome-specific TL shortening was observed at mid-chemo for 1p (p =0.022), 5q (p = 0.041), 7q (p =0.025), 9q (p =0.045), 18q (p =0.002), 20p (p =0.020), 21q (p =0.040) and 22p (p =0.025). Type of chemotherapy was shown to be a significant predictor of both mean TLs (TAC significantly greater than TCH; p= 0.036) and chromosome-specific TLs for 32 of the 46 chromosomal arms (p=0.004 to 0.049*).* Pain was found to be a significant negative predictor of chromosome-specific TLs (higher pain; shorter telomeres) for 5q (p=0.040), 8p (p=0.047), 13p (p=0.019), 20p (p=0.036), 22p (p=0.035), Xp (p=0.014), Xq (p=0.039). Expanding upon the knowledge gained from this study offers hope for the future development of biomarkers that could identify patients at risk for PNS and improve their QOL.

Chapter 1

General Background

Breakthroughs in Early Telomere Research

Telomeres were first described in the 1930s by Hermann Muller and Barbara McClintock, through their studies in Drosophila (Muller, 1938) and maize (McClintok, 1941), as special structures at the end of chromosomes required for their integrity. Muller first coined the term "telomere" and three years later, McClintock (1941) proposed that telomeres stabilize chromosome ends and prevent them from being recognized as DNA double strand breaks.

In 1961, Leonard Hayflick and Paul Moorhead developed a theory that cells cultured *in vitro* were not immortal, but could replicate only a finite number of times, a phenomenon now known as replicative senescence or the the "Hayflick limit", and suggested the existence of a cellular counting mechanism (Hayflick & Moorhead, 1961). Furthermore, they observed the presence of two broad classes of cells, normal mortal cell strains and immortal cancer cell lines (reviewed in Hayflick, 1998). Their discovery challenged a central dogma of the time that supported the belief that cultured cells would divide indefinitely *in vitro*, with any culture failures being attributable simply to a lack of technical expertise.

Fast forwarding to the 1970s, James Watson described the cell's inability to replicate up to the tip of the chromosome as the "end replication problem" (Watson, 1972). By combining the knowledge that the properties of DNA polymerase prevent cells from fully replicating the ends of linear chromosomes with the observation that normal cells have a limited capacity to replicate,

Olovnikov, was the first to suggest that chromosomal shortening may be linked to a cell's eventual mortality (Olovnikov, 1973). It has been reported that Olovnikov developed this hypothesis while waiting for a subway train in Moscow. As he heard the train coming, he imagined the train, and specifically the engine, being the DNA polymerase and the track being the DNA. The engine (DNA polymerase) would not be able to replicate the first segment of DNA (the track) because it lays exactly underneath the engine. It seemed unlikely that with each cell division a DNA segment containing important genes was lost. Therefore, Olovnikov reasoned that the repeated non-coding telomeric nucleotide sequences act as a buffer to protect gene coding sequences. He correctly speculated that with each round of cell division a portion of the telomere "buffer" would be lost, and that the length of the telomeric "buffer" could be important for determining a cell's ability to proliferate (reviewed in Hayflick, 1998; Greider, 1998).

In 2009 the Nobel Prize in Medicine or Physiology was jointly awarded to Elizabeth Blackburn, Carol Greider and Jack Szostak for elucidating the structure and maintenance of telomeres. The work leading to this prize dates back to 1978 when Blackburn, together with her mentor Joseph Gal discovered the telomere's DNA sequence (Blackburn & Gall, 1978). In collaboration with Jack Szostak, Blackburn published evidence for the evolutionary conservation of telomeres and their role in providing chromosomal stability (Szostak & Blackburn, 1982). Furthermore, insight to the regulation of telomere length was gained by Greider and Blackburn in 1983 by discovering enzymatic activity in Tetrahynema cell extracts that compensated the incomplete replication in telomere ends (Greider & Blackburn, 1985). They subsequently purified the enzyme and found it to be a ribonucleoprotein (RNP) that was comprised of an RNA component, as well as a protein component. Thus, they had discovered a new type of DNA

polymerase, which they named “telomerase” (Greider & Blackburn, 1987). Moreover, they discovered that the RNA component contained the template required for the addition of nucleotide repeats onto telomeres. Although all of these findings were in Tetrahymena, it is now known that telomerase provides the main mechanism by which telomeres are elongated in eukaryotes. Taken together, these discoveries were the foundation for the importance of telomere biology in normal aging and in pathology.

Structure of the Telomeric Region

As a result of intensive research that has been completed since these pioneering studies, much is now known about the telomeres. In humans, telomeres are stretches of non-coding tandemly repeated “TTAGGG” DNA sequences that can reach a length of 15,000 base pairs. They are located at the terminal ends of all vertebrate chromosomes, including those of humans (Moyzis et al., 1988). Approximately 50-300 base pairs of single stranded DNA that is G-rich extends from the 3’ strand forming an overhang that is thought to be important for telomere function (Makarov et al., 1997, Wright et al., 1997). This single stranded 3’ overhang folds back on itself forming a large loop structure called a telomere loop or T-loop, that has a shape similar to that of a paper clip, and invades an upstream telomeric double stranded DNA forming a displacement loop or D-loop.

Telomere stabilization and maintenance relies on a six-protein complex called shelterin, which is composed of telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), the human ortholog of the yeast repressor/activator protein 1 (Rap1), TRF1 interacting nuclear factor 2 (TIN2), protection of telomerase 1 (POT1) and TPP1. Shelterin components specifically localize

to the telomere due to the recognition of the duplex part by TRF1 and TRF2 and their interacting proteins Rap1 and TIN2, whereas POT1 forms a heterodimer with TPP1 and recognizes the G-strand overhang. These protein-DNA and protein-protein interactions are responsible for maintaining telomere homeostasis (Palm & de Lange, 2008).

The chromatin structure of mammalian telomeres has properties that are characteristic of heterochromatin. In particular, trimethylation of histone H3 lysine 9 (H3K9) and histone H4 lysine 20 (H4K20) are observed in telomeric and sub-telomeric regions (reviewed in Blasco, 2007). In addition, these regions are enriched in heterochromatin protein 1 (HP1), which is important for chromatin compaction (reviewed in Blasco, 2007). Another mark of heterochromatin that is present in telomeric and sub-telomeric regions is low levels of acetylated H3 (AcH3) and H4 (AcH4) (reviewed in Blasco, 2007). Also, akin to heterochromatic regions, the sub-telomeric regions are gene-poor, and the telomeric regions do not contain any genes (reviewed in Blasco, 2007).

Telomere Functions

The telomere serves to cap chromosome ends and maintain genomic stability through various mechanisms. One function is to prevent chromosomal ends from being distinguished as DNA double strand breaks; thereby avoiding the activation of DNA damage signaling pathways and genome instability (de Lange, 2009). Ataxia telangiectasia mutated (ATM) kinase pathway and ataxia telangiectasia and Rad3 related (ATR) kinase pathway are activated in mammalian cells in response to DNA double and single strand breaks in order to arrest the cell cycle and/or induce apoptosis (programmed cell death). Moreover, homology directed repair (HDR) and non-

homologous end joining (NHEJ) repair pathways can also be activated to ensure that cells continue dividing with an intact genome. Telomeres, along with the shelterin complex, offsets the effect of these four "repair" processes. Specifically, the TRF2 subunit of shelterin is responsible for ensuring that the ATM kinase signaling pathway and NHEJ repair pathway are not activated by formation of the T-loop. The POT1 subunit is responsible for repressing the ATR kinase signaling pathway by preventing RPA, the single stranded DNA binding protein, from binding to the telomeric single stranded DNA. Both TRF2 and POT1 block the activation of the HDR pathway. Failure to do so causes cell cycle arrest under the direction of: a) ATM and/or ATR; b) chromosome end-to-end fusion mediated by NHEJ; or c) sequence exchange by HDR involving two telomeres or a telomere and another part of the genome (de Lange, 2002, d'Adda di Fagagna et al., 2003, Bailey & Murnane, 2006).

Due to the inherent inability of the DNA machinery to fully replicate the end of linear chromosomes, successive cell division leads to attrition of chromosome ends. Given that, telomeres are capable of averting the end replication problem and avoiding the loss of valuable genetic information by the presence of the cellular ribonucleoprotein enzyme telomerase, which can add telomeric repeat sequences to the end of chromosomes (de Lange, 2009). Additionally, telomere attrition can be compensated for through a recombination based telomerase-independent mechanism termed alternative lengthening of telomeres (ALT) (Xu et al., 2013).

Regulation of Telomere Length

In humans, telomere length is maintained by the the enzyme telomerase, which is composed of a reverse transcriptase protein (TERT) consisting of 1132 amino acids encoded by the hTERT

gene, which is located on the short arm of chromosome 5 (5p15.33). Additionally, it is composed of telomerase RNA component containing 451 nucleotides, which is encoded by the telomerase RNA gene hTERC, which is located on the long arm of chromosome 3 (3q21-q28) (reviewed in Aubert & Lansdorp, 2008). TERT uses the telomerase RNA component (that is complementary to the telomere sequence) as a template for telomere repeat synthesis at the 3' overhang. Although telomerase is expressed during development, post-neonatal somatic cells repress telomerase expression except in germ cells, stem cells and a subset of somatic cells (such as active fibroblasts). Most neoplastic cells derepress the expression of hTERT to allow for immortalization and tumor formation (reviewed in Cesare & Reddel, 2008).

As stated above, elongation of telomeres in cells not depending on telomerase is called ALT. To date, most of the molecular details in ALT pathways remain unknown and very little is known regarding the mechanism(s) by which this pathway is activated. Current findings suggest that this process relies on homologous recombination between individual telomeres, where one telomere serves as a template for recombination-based elongation of another telomere (Gocha et al., 2013).

Telomere Shortening, Aging, and Disease

A lack of telomerase activity in the majority of normal cells is associated with the end replication problem resulting in the loss of 50-100 base pairs at the telomeric end after each round of cell division (Oeseburg & de Boer, 2010). This mechanism functions as a biological clock to eliminate cells that have lived too long or long enough to accumulate aberrations and mutations. Following approximately 50-70 cell doublings, gradual telomere shortening

eventually results in critically short telomeres, leading to the activation of a DNA damage response (DDR) and the cell enters a state of permanent growth arrest (senescence) or apoptosis (Oeseburg & de Boer, 2010).

In humans, twin studies have concluded that telomere length is highly heritable, with inheritance estimates ranging from 40% up to 80% (Slagboom et al., 1994, Vasa-Nicotera et al., 2005, Andrew et al., 2006, Najajou et al., 2007). While genetic determination of telomere length was thought to be X-linked, current evidence suggests paternal inheritance (Nawrot et al., 2004, Nordfjall et al., 2005). At birth, one study found no difference in telomere length between sexes in newborn (Okuda et al., 2002). Another study found that female newborns have longer telomere lengths than males (Aubert et al., 2012). Moreover, adult males have been found to have shorter telomere lengths than adult females suggesting an influence of environmental factors and/or estrogen (Okuda et al., 2002, Hunt et al., 2008). Racial differences have also been found to impact telomere length. In newborns as well as in adults, African-Americans have been reported to have longer telomeres than Caucasians (Zanni & Wick, 2011, Drury et al., 2015).

Telomere attrition is one of the well-known cell intrinsic events associated with normal cellular aging, both directly and indirectly. Cellular senescence is defined as a state where the cell is unable to proliferate even with the availability of nutrients and mitogens (Campisi & d'Adda di Fagagna, 2007). Given that aging is characterized by an increase in senescent cells in many tissues and the observation of an inverse relationship between age and telomere length, replicative senescence induced directly through telomere shortening is involved in the aging process by limiting tissue renewal and influencing tissue homeostasis (Shay & Wright, 2005). Although the physiological role of senescent cells is still poorly understood, these cells secrete

pro-inflammatory cytokines, thereby negatively affecting the tissue microenvironment and function (Collado, Blasco & Serrano, 2007).

In addition, oxidative stress has been implicated in triggering cellular senescence. Harman's "free radical theory" is one of the oldest of current theories suggesting that damage to cellular macromolecules via free radicals is a major determinant of life span. Oxidative stress can be defined as the net result of an imbalance between production and destruction of reactive oxygen species (ROS) (reviewed in Kregel and Zhang, 2007). Because of it's high content of guanine bases, telomeric DNA is chemically more prone to oxidative damage, which, in turn, accelerates telomere shortening (reviewed in Blackburn, Epil & Lin, 2015). Therefore, the notion of telomeres acting as a cumulative measure of oxidative stress suggests an indirect involvement of accelerated telomere attrition with normal cellular aging.

More importantly, telomere attrition and dysfunction have been shown to be a causal factor in the acquisition of age-related diseases, including but not limited to atherosclerosis (Bentos et al., 2004, de Beer et al., 2015); myocardial infarction (Brouilette et al., 2003, D'Mello et al., 2016); Alzheimer's dementia (Panossian et al., 2003, Guan et al., 2013); and heart failure (reviewed in Oeseburg., 2010). Accelerated telomere shortening has also been associated with a wide array of lifestyle factors such as smoking, consumption of unhealthy diet, obesity and lack of exercise (reviewed in Shammas, 2011). Furthermore, telomeres are implicated in cancer biology, due to their stabilizing effect on the chromosome and association with cellular proliferation (de Lange, 2005, Xu et al., 2013). The progression from a normal cell to a cancer cell occurs through a number of effects in which the cell acquires the so-called "hallmarks of cancer" (Hanahan & Weinberg, 2000). Replicative telomere attrition is considered one of several

regulatory mechanisms that blocks cell immortality, which is considered as one of the hallmarks of cancer cells (reviewed in Xu et al., 2013). Replicative senescence occurs in a cell following the activation of ATM and ATR signaling pathways and the associated downstream factors including CHK1, CHK2, and phosphorylation of p53 and results in a cell cycle arrest in Gap 1 (G1) phase of the cell cycle, thereby inhibiting tumorigenesis (reviewed in Raynaud et al., 2008). However, a DNA damage checkpoint deficient cell can continue dividing through p53 or pRb inactivation, resulting in extensive telomere attrition. As a result, telomeres lose their protective function, thereby allowing frequent chromosomal fusions and rearrangements, which, in turn, leads to massive genomic instability, the latter of which can promote carcinogenesis. In addition, cell death is bypassed and immortality is achieved by telomere length maintenance through the up-regulation of telomerase in > 85% of human tumors (Raynaud et al., 2008). In the remaining ~15% of human tumors, telomere length is maintained by ALT (reviewed in Cesare & Reddel, 2008).

Impact of Environmental Factors on Telomere Length

Variation in the rate of biological aging reflects the accumulative burden of genetic, metabolic and environmental stressors, resulting in oxidative damage and elevated inflammatory processes (Lamb and Shiels, 2009). Stress results in the activation of the hypothalamic pituitary adrenal (HPA) axis and the release of glucocorticoid hormones by the adrenal glands. These hormones have been associated with reduced antioxidant capacity that may therefore cause increased oxidative damage to the DNA and accelerate telomere shortening (Patel et al., 2002, von Zglinicki, 2002, Irie at al., 2003). Telomere length have also been linked with various psychological conditions (Lindqvist et al., 2015). Shorter telomere length has also been linked

with longer duration of chronic stress (mothers caring for chronically ill children) in comparison to mothers caring for healthy children with a difference equivalent to 10 years of life between the two groups (Epel et al., 2004). Based on this observation, it could be hypothesized that women under stress were at an increased risk of developing age-related health problems. In a similar study, investigators reported higher depressive symptoms and shorter telomere lengths among caregivers of patients with Alzheimer disease compared to gender and age-matched controls (Damjanovic et al., 2007).

Beside psychological conditions, lifestyle behaviors have also been shown to impact telomere length. Smoking is associated with accelerated telomere attrition through the increase in oxidative stress with a pack of cigarettes a day for a period of 40 years being equivalent to 7.4 years of life (Valdes et al., 2005, Song et al., 2010, Babizhayev et al., 2011). Obesity also has a negative impact on telomere length through obesity-related oxidative damage and the deregulated production of adipocytokines (Valdes et al., 2005, Song et al., 2010). Exposure to genotoxic agents that cause damage to the DNA and its association with telomere length have also been evaluated. Chemotherapeutic drugs have been shown to have a negative impact on telomere length by means of oxidative stress and uncapping telomeres (Lu et al., 2013, Liu, Hales and Robaire, 2014). Radiation-induced telomere dysfunction has also been reported by causing DNA double strand breaks, which are inefficiently repaired at the telomere or by telomere uncapping in human cancers (Li et al., 2012, Mirjolet et al., 2015). However, the effect of radiation on telomere dynamics has not been thoroughly studied. Investigators showed shorter telomere length in traffic police officers exposed to toluene and benzene pollution, relative to telomere length in office police workers (Hoxha et al., 2009). Similarly, significantly increased chromosomal instability and shorter telomere length have been noted in coke-oven workers

exposed to polycyclic aromatic hydrocarbons, relative to matched controls (Pavanello et al., 2010). Furthermore, lower socio-economic status is associated with a decline in telomere length. It is assumed that low income increases the likelihood of being on a poor quality diet, fat and sugar rich, which are known to result in the production of more ROS(s) that directly causes DNA breaks (Saretzki & VonZglinicki, 2002, Sheils et al., 2011). Also, the results of several studies support associations of short sleep duration and poor quality sleep with shorter leukocyte telomere length (Prather et al., 2015, reviewed in Tempaku, Mazzotti & Tufik, 2015).

In contrast, healthy lifestyle behaviors that decrease oxidative stress and inflammation have been shown to preserve telomere length and reduce the pace of aging. Telomere length correlates positively with the increased dietary intake of fiber (Cassidy et al., 2010). Moreover, a diet rich in antioxidants, particularly, omega 3 fatty-acids is associated with reduced rate of telomere shortening in participants of a study over a period of 5 years (Farzaneh-Far et al., 2010). In addition, longer periods of physical activity and intensive sports have been associated with longer leukocyte telomere lengths especially among older adults (Sabenroth et al., 2015, Soares-Miranda et al., 2015). A study in 2,401 healthy twins has shown that physically active participants have longer leukocyte telomere length when compared with their age and gender-matched non-exercisers (Cherkas et al., 2008). Psychosocial stress reduction through mindfulness meditation was not only found to increase telomerase activity in peripheral blood mononuclear cells but also lengthen telomeres (Schutte & Malouff, 2014, Carlson et al., 2015).

Telomere Length Measuring Techniques

There is a large and rapidly growing body of data showing the implication of telomere biology in aging and disease processes. Indeed, telomere lengths have emerged as a potential biomarker for risk assessment of diverse health outcomes (Fossel, 2012). The "gold standard" method for measuring telomere length is the terminal restriction fragment (TRF) technique by southern blot (Southern, 1979). Genomic DNA is typically digested using a cocktail of 4-6 restriction enzymes that lack recognition sites in telomeric and subtelomeric regions. The intact telomere fragments are then visualized and size estimated via gel electrophoresis. In addition to providing only an average of total telomere lengths, shortcomings of this assay include the need for large amounts of DNA (micrograms), as well as the technique being labor intensive. Also, inclusion of subtelomeric regions in this assay, due to the nature of the restriction enzymes used, can provide an overestimation of the true telomere length (reviewed in Montpetit et al., 2014).

Polymerase Chain Reaction (PCR)-based techniques have also been used in measuring telomere length by amplifying both telomere repeats and a single copy housekeeping gene(s) (Cawthon, 2002, Cawthon, 2009). Calculating the ratio between the two quantities yields a measure of relative telomere length (Cawthon, 2002, Cawthon, 2009). PCR-based methods include quantitative PCR (qPCR), monochrome multiplex qPCR (MMqPCR), and absolute telomere length (aTL) qPCR. Unlike the TRF assay, PCR- based telomere measuring assays require small amounts of DNA (nanograms) and are considered high-throughput assays of relatively low cost. However, like TRF, PCR-based methodologies do not provide information

on telomere length at individual chromosome ends; hence they do not allow for the recognition of specific chromosomes with short and/or dysfunctional telomeres (reviewed in Vera & Blasco, 2012).

Single telomere length analysis (STELA) is a telomere measuring assay that overcomes the limitations of TRF and qPCR based methods by providing a length for specific individual chromosomes (Baird et al., 2003). STELA is a qPCR-based technique that is designed to amplify the telomeric DNA of a single chromosome, using primers that are specific for the sub-telomeric sequence of that chromosome. Measuring telomere lengths on individual chromosomes is of great importance since a single or a small number of short telomere(s) has (have) been suggested to initiate a DNA damage response leading to senescence or apoptosis (Hemann et al., 2001, Abdallah et al., 2009). Unfortunately, to date, telomeres of only a subset of chromosomes (Xp, Yp, 2p, 11q, 12q, and 17p) can be measured through this technique due to the lack in specificity of sub-telomeric regions between chromosomes. Another major disadvantage of this technique is its limited ability for the analysis of very long telomeres (reviewed in Montpetit et al., 2014).

One of the most accurate telomere measuring methods is the Quantitative Fluorescence In Situ Hybridization (Q-FISH) based assay (Lansdorp et al., 1996). Q-FISH of telomeric repeats is assessed following hybridization with a fluorescent $(CCCTAA)_3$ probe, with the remaining chromatin on the chromosome being visualized by a nonspecific DNA stain such as DAPI or propidium iodide (Krejci & Koch, 1998). Peptide Nucleic Acid (PNA) probes used in this assay have higher hybridization efficiency to telomeric repeats than DNA probes because of their neutral (uncharged) backbone (Egholm et al., 1993). Furthermore, PNA probes show increased thermal stability of the probe-target duplex. Despite being labor intensive and expensive, this

high resolution technique allows the quantification of each of the individual 92 human telomeres. It is also powerful in detecting chromosomes with telomere free ends (< 0.5 kb) (reviewed in Aubert, Hills & Lansdorp, 2012).

Chapter 2

Introduction

Telomeres, which are crucial in maintaining genomic integrity and preventing chromosomal instability have been suggested to play a role in carcinogenesis (Artandi & DePinho, 2010). Among the different types of cancer, several studies have been conducted to investigate the association between telomere length and breast cancer risk (Table 1; reviewed in Zhu et al., 2016). Breast cancer (BC) is a very heterogeneous disease, both genetically and clinically, that forms in tissues of the breast (Stingl & Caldas, 2007). A classification system has been developed in order to standardize the disease and provide insight for better treatment and prognosis strategies. Histologically, BC is categorized into in situ carcinoma and invasive carcinoma (Malhotra et al., 2010). The two main subtypes of breast carcinoma in situ are ductal carcinoma in situ (DCIS) (forms in tubes that carry milk to the nipple) and lobular carcinoma in situ (LCIS) (forms in glands that produce milk) (Malhotra et al., 2010). Invasive breast carcinoma forms in the ducts or lobules and spreads to other tissues of the breast, and is also further subdivided into invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). Immunohistochemistry/FISH tests have been used to provide a molecular characterization of BC. Key prognostic categories that have emerged are based on the expression status of the estrogen (ER) receptors, progesterone (PR) receptors and/or human epidermal growth factor receptor-2 (HER2) status (Westbrook & Stearns, 2013). Estrogen receptor (ER) positive cancer refers to BC in which the cells have an increased sensitivity to estrogen leading to cancer tumor growth. HER2 positive breast cancer has an amplification (too many copies) of the HER2 gene and/or increased expression of the HER2 protein, which causes the tumor to grow faster and the cancer

to be more aggressive. Microarray-based gene expression analyses have further identified molecular subtypes of BC as: basal like (ER-, PR-, and HER2-; also described as triple negative), luminal subtype A (ER+ and/or PR+; HER2-, + or - Ki-67), luminal subtype B (ER+ and/or PR+; HER2+, + or - Ki-67), and HER2 enriched (ER-, PR- and HER2+) (Perou et al., 2000, Sorlie et al., 2001, Sorlie et al., 2003). In addition to the prognostic value of histopathological classifications of BC, the identification of molecular subtypes enabled the prediction of response to newer targeted therapies.

Clinical and histopathological factors, including tumor stage, tumor size and nodal status, along with the growing knowledge of molecular biomarkers, have advanced the process of choosing effective and safe treatment options for women with BC. Surgical courses of action in operable tumors include breast-conserving surgery (also called lumpectomy) for early-stage BC or mastectomy for advanced BC (Myers, 2016). Neoadjuvant approaches (chemotherapy and hormonal therapy prior to surgery) for selected high-risk BC aims at shrinking the tumor size to potentially reducing the extent of surgery (Thompson & Moulder-Thompson, 2012). Adjuvant systemic therapy is a standard of care after surgery to eradicate possible micrometastatic tumor and lower recurrence risk in early-stage disease through the use of chemotherapy and hormonal therapy, whereas in advanced stage BC, it serves to control disease burden and improve survival (Myers, 2016). In the case of ER positive BC, which accounts for approximately 80% of BC, hormonal therapy is the initial treatment of choice in the form of selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene or aromatase inhibitors (AIs) (Westbrook & Stearns, 2013). HER2 targeted therapies include trastuzumab, pertuzumab, ado-trastuzumab emtansine, and lapatinib. Trastuzumab, which is the most commonly used therapeutic agent for women with HER2+ tumors, uses a monoclonal antibody that binds to the HER2 receptor,

thereby suppressing cell proliferation (Giordano, Tagliabue & Pupa, 2012). Triple negative breast cancer is considered aggressive, with no targeted therapy available. Therefore, cytotoxic chemotherapy is an option offered for patients with this type of BC (Myers, 2016). There are several classes of cytotoxic chemotherapeutic agents, including anti-metabolites, anti-microtubules, anthracyclines and cychlophosphamide (Westbrook & Stearns, 2013). One example of an anti-metabolite is capecitabine, which slows tumor growth by inhibiting DNA synthesis during the S phase of the cell cycle within the tumor (Parker, 2010). Among the most effective of the antimicrotubules treatments are the taxanes paclitaxel (taxol) and docetaxel (taxotere) (Westbrook & Stearns, 2013). These drugs inhibit microtubule formation, leading to mitotic arrest and suppression of cell growth (Fauzee, 2011). The anthracyclines doxorubicin (adriamycin) and epirubicin have been extensively used in the course of treatment for BC (Quiles et al., 2002). Several mechanisms have been proposed for doxorubicin-mediated cell death, including topoisomerase II poisoning, DNA adduct formation, oxidative stress and ceramide overproduction (Gewirtz, 1999, Minotti et al., 2004, Senchenkov, Litvak & Cabot, 2001, Yang et al., 2014).

Cancer is a leading cause of death in both more and less economically developed countries. In 2013, there were 14.9 million incident cancer cases worldwide. Of these, BC was the leading cause for cancer incidence among women, accounting for 1.8 million of those cases (Global Burden of Disease Cancer Collaboration, 2015). In 2016, approximately 246,660 women are expected to be diagnosed with BC and 40,450 deaths from BC are estimated in the United States (Siegel, Miller & Jemal, 2016). However, since the year 2000, BC mortality rates decreased significantly due to the availability of better screening tools, advanced detection strategies, and continually improving treatment options (Jemal et al., 2013). Although these factors have

increased the survival rate to at least five years in 90% of women receiving an early diagnosis, many cancer survivors have reported the occurrence of a number of distressing symptoms leading to long term effects that adversely affect their quality of life (QOL) (Jemal et al., 2009, Dodd et al., 2010). These symptoms, which manifest from the cancer itself or it's treatment include (but are not limited to): anxiety, fatigue, depression, pain, stress, and sleep disturbances, which we are collectively categorizing as "psychoneurological symptoms" (PNS) (Lee at al., 2004, Badger et al., 2007, Bardwell & Ancoli-Israel, 2008, Bower, 2008, Weinberger et al., 2010, Satija et al., 2014, Jung et al., 2016). PNS not only impact treatment decisions in patients with BC but can also challenge the cancer survivor's ability to perform and maintain employment, connect with loved ones and carrying on day-to-day tasks (Hurria, Somlo & Ahles, 2007). Although there is increased awareness to the importance of understanding the biological basis of PNS, the molecular mechanism(s) leading to the development and persistence of PNS remains inconclusive.

One reason patients may present with a number of symptoms, rather than a single symptom, is that these symptom "clusters" may share a common biological mechanism (Miaskowski & Aouizerat, 2007). Several studies have been conducted in order to understand the biological mechanism(s) underlying the development of PNS. Symptoms of cancer/cancer treatment are thought to be due, at least in part, to cytokine-induced sickness behavior (Cleeland et al., 2003). Cytokines are present in increased levels in patients with cancer (Meyers et al., 2005) and can be produced from tumor tissues (Sasaki et al., 2001) or in both tumor tissues and healthy cells in response to chemotherapy (Pusztai et al., 2004). Although cytokine-induced sickness behavior is considered a reasonable mechanism leading to symptom clustering in patients with cancer, it can not account for all the clusters reported to date; the reason being that several investigators have

reported different numbers of symptoms in a cluster, as well as different types of symptoms within a cluster (Miaskowski and Aouizerat, 2003). Several investigators have shown an association between each of the PNS and the activation of inflammation markers, particularly pro-inflammatory cytokines (reviewed in Kim et al., 2012). However, these studies have been limited because they focused on single symptoms and single markers of inflammation, and they haven't clearly explained the mechanism leading to the development and persistence of PNS. A higher than expected incidence of some PNS as well as pro-inflammatory cytokines, have been reported prior to the initiation of chemotherapy (Meyers et al., 2005). This suggests that inflammation may be activated by the cancer itself, with the symptoms being worsened by chemotherapy. In addition, it has been suggested that some of these PNS, such as depression and fatigue, are actually caused by perturbations in cytokine levels acting on the brain (Dantzer et al., 2008). However, the fact that inflammatory molecules, such as cytokines, are not retained for long periods of time, and the fact that these symptoms have been reported to persist in a subset of cancer survivors for years after therapy, led us to hypothesize that inflammation is an initiating step for a cascade of biological events that also include the acquisition of somatic cell genetic (e.g. telomere length changes; chromosomal instability) and/or epigenetic changes (e. g. methylation alterations), with these latter genetic/epigenetic alterations providing a means for the effects of the BC and/or its treatments to be maintained in one's biological memory.

Acquired telomere attrition has long been speculated as a causal factor in the acquisition of many chronic diseases (Blackburn, Epil and Lin, 2015). Furthermore, accelerated telomere shortening has been shown to play a role in the pathogenesis of multiple psychological conditions such as, but not limited to, stress, depression and cognitive decline, all of which are major side effects experienced by patients with BC (reviewed in Starkweather et al., 2014).

Telomere shortening can be accelerated by multiple factors associated with the cancer itself or it's treatment including (but not limited to) chemotherapy and oxidative damage (von Zglinicki, 2002, Li et al., 2012). Interestingly, increased inflammatory activity can accelerate telomere attrition by inducing cell turnover and replicative senescence or by the release of ROSs that shorten and damage telomeres through oxidative stress (Aviv, 2004, Jaiswal et al., 2000). Induced cellular proliferation/senescence could contribute to PNS, at least in part, through compromised tissue homeostasis. In addition, shortening of telomeres has been associated with an increased frequency of chromosomal instability, which, in turn, could give rise to alterations in gene expression, thereby allowing the persistence of PNS. Furthermore, a reduction in the telomere heterochromatic region as a result of telomere attrition could alter gene expression of adjacent subtelomeric genes through a phenomenon known as "telomere position effect" (TPF) (van Deutekom et al., 1996, Baur et al., 2001, Robin et al., 2014). One can speculate that an epigenetic alteration in the genes juxtaposed to the telomeric region, could provide a plausible mechanism for the persistence of PNS into survivorship.

As noted earlier, telomere length alterations in patients with BC have been evaluated extensively as a diagnostic marker in breast tissues, but few investigators have incorporated the effect of chemotherapy on the telomere lengths of healthy, peripheral tissues in these patients (Meeker et al., 2004). However, research involving measuring telomere length in peripheral blood lymphocytes has been completed, with a focus on investigating the potential link between telomere length and the risk for developing BC or other age-related diseases (Table 1) (Heaphy & Meeker, 2011, Barrett et al., 2015). The results of one study on leukocytes obtained from patients with early stage BC showed decreased telomere length at five and nine months following chemotherapy when compared to baseline values (Schroder et al., 2001). However,

information regarding PNS was not integrated into that study since the main focus was to determine the effect of stem cell transplantation following chemotherapy in the evaluated group of patients.

Given that telomere shortening is accelerated by inflammation and oxidative stress, and that chemotherapy affects both of these processes, the primary objective of this study was to longitudinally evaluate telomere length in women diagnosed with early stage BC who were treated with chemotherapy in order to **determine if chemotherapy contributes to chromatin breakage within the telomere, thereby leading to telomere shortening** in this group of patients. Results from this study will allow us to test the following hypotheses:

1. There is a change in telomere lengths following treatment with chemotherapy in women diagnosed with early stage BC.

2. These differences in telomere lengths affect a subset of chromosomal arms rather than affecting all chromosomes equally.

3. The extent of change in telomere length differs based on the type of chemotherapy regimen used for treatment.

4. A correlation exists between telomere length changes following treatment for BC and a survivor's acquisition of PNS.

To our knowledge, this is the first longitudinal study that provides information about telomere lengths over five time points, including pre-, during and post-chemotherapy treatment. Moreover, it is the first study to provide information about chromosome-specific telomere length

changes associated with chemotherapy. These expanded observations are expected to provide new insights in the use of peripheral blood telomere length as a biomarker for the identification of individuals who are sensitive to a specific type of chemotherapy and/or at higher risk for the development of PNS.

Table 1: Studies on the Association Between Telomere Length and Risk for BC

Reference	Year	No. of Case/Control	Sample source	Method	Study outcome
Barewell et al.	2007	72/1696	Leukocyte	TRF	TRF derived telomere length is not a marker for BC susceptibility
Shen et al.	2007	283/347	Leukocyte	qPCR	Modest evidence that short telomere length is associated with increased breast cancer risk
Shen et al.	2009	1026/1070	Leukocytes	qPCR	Short telomere length is associated with breast cancer risk in pre-menopausal women and women with low antioxidant dietary or supplementary intake
De Vivo et al.	2009	896/917	Leukocytes	qPCR	No significant association between postmenopausal BC risk and short telomere length
Zheng et al.	2010	293/335	Leukocytes	Q-FISH	Increased risk of BC with shorter telomere length on chromosome 9p, 15p, 15q and Xp
Gramatges et al.	2010	102/50	Leukocytes	qPCR	Longer telomere length in women at high risk for BC based on family history
Kim et al.	2011	342/735	Leukocytes	qPCR	No association between telomere length and BC risk
Qu et al.	2013	601/695	Leukocytes	qPCR	Shortened telomeres is associated with increased risk of BC
Pellatt et al.	2013	728/720	Leukocytes	qPCR	Longer telomere length is associated with increased BC risk

Adapted from reviewed in Zhu et al., 2016.

Chapter 3

Materials and Methods

Study Participants Ascertainment

A total of 77 women with early stage (I to IIIA) breast cancer were ascertained through the Massey Cancer Center (MCC) in Virginia Commonwealth University and its multiple collaborative sites, including Hematology Oncology Associates of Fredericksburg, Inc., Bon Secours Richmond Health System, Peninsula Cancer Institute, and Rappahannock General Hospital. To identify potential study participants, a member of the research team attended the weekly interdisciplinary Breast Health meetings at Virginia Commonwealth University Health System (VCUHS). The eligibility criteria were: 1) an age of 21 years or older; 2) a diagnosis of early stage BC with a scheduled visit to receive chemotherapy; and 3) a female gender. Exclusion criteria were a history of: 1) a previous cancer, or chemotherapy; 2) a diagnosis of dementia; 3) active psychosis; or 4) immune-related diagnosis (e.g. multiple sclerosis; systemic lupus erythematous). From the list of patients presented at the meeting, those persons meeting the study inclusion criteria were identified. The study coordinator then consulted with the attending oncologist and with his/her approval approached the patient regarding her desires to participate in the study. The offsite research nurses followed similar procedures for identifying potential participants at his/her respective site.

After providing their informed consent (VCU IRB # HM 13194), all participants in this longitudinal study completed questionnaires and cognitive testing via a computerized system at each of the five time points, which were: 1) visit 1 (also referred to as “baseline”) which was

prior to chemotherapy; 2) visit 2 (also referred to as "mid-chemo"), which was prior to the fourth cycle of chemotherapy; 3) visit 3, which was scheduled approximately 6 months following the initiation of chemotherapy, at which time a subset of women received radiotherapy; 4) visit 4, which was scheduled approximately 1 year following the initiation of chemotherapy; and 5) visit 5, which was scheduled approximately 2 years following the initiation of chemotherapy. During each visit, blood samples (in green top (sodium heparin) tubes and lavender (EDTA) tubes) were collected by venipuncture or an existing access device and transported to the cytogenetics laboratory in a biohazard container. The lab staff were blinded to the clinical history and therapy status of the study participants at the time of sample processing and evaluation.

Demographic, Lifestyle and Clinical Health Information

Demographic and lifestyle information was obtained from each study participant via self reporting questionnaires. The subset of the demographic data selected as most relevant for this current study included age, race and income. Clinical health information was obtained from medical records. This information included BC stage, BC grade, chemotherapy regimens and tumor characteristics. Tumors were characterized based on FISH and/or immunohistochemical testing results as luminal A (ER+ and/or PR+; HER2- ;no information was available on Ki-67), luminal B (ER+ and/or PR+; HER2+; no information was available on Ki-67), HER2 positive (ER-, PR- and HER2+) and triple negative (ER-, PR-, and HER2-) (Perou et al., 2000, Sorlie et al., 2001, Sorlie et al., 2003).

Treatment

Chemotherapy treatment for each study participant was administered as either: 1) Taxotere (docetaxel), Adriamycin (doxorubicin), and Cyclophosphamide [TAC]; 2) Taxotere and Cyclophosphamide [TC]; or 3) Taxotere (docetaxel), Carboplatin (paraplatin) and Herceptin (transtuzumab) [TCH]. Information regarding chemotherapy and radiation therapy for each patient was determined from medical records.

DNA Isolation

Genomic DNA was extracted from whole blood using a Puregene DNA Isolation Kit (Qiagen) according to the manufacturers protocol. After extraction, the DNA was quantified, evaluated to ensure it was not degraded, and stored at -80°C. DNA stocks were diluted to approximately 20 ng into pure water prior to setting up qPCR runs.

Monochrome Multiplex qPCR (MMqPCR)

The relative genomic telomere length was measured by monochrome multiplex real-time quantitative PCR (MMqPCR) as previously described, in which both the telomere and the housekeeping gene values are derived from the same well (Cawthon, 2009). The primer sequences used for this assay are shown in Table 2. In this method, relative telomere length is determined as a ratio of the number of copies of the telomere repeat sequences compared to the copies of a single housekeeping gene (human albumin), with this number being derived from a standard curve. This 5-point standard curve was derived from triplicate measures on each assay plate. DNA for the standard curve was made from a "cocktail" of 11 healthy control women and

was prepared in dilutions ranging from 1.235 to 100 ng. PCR reactions were set up by aliquoting 5 µL of 4 ng experimental DNA followed by 15 µL of SYBR Green Mastermix combined with telomere primer and albumin primer into each reaction well of a 96-well plate. Each plate was set up to include "standard DNA" in triplicate; experimental DNA in triplicate; a triplicate of a negative control (lacking DNA); and a triplicate of a positive control, which is DNA extracted from HeLa cells (known to have high T/S ratio), a normal female control age 35 cells (known to have medium T/S) ratio and MCF7 cells (known to have low T/S ratio). All 5 visits for each study participant were run on the same plate. This assured that comparisons between the five time points were always within the same batch, thus eliminating possible biases due to batch differences. An additional layer of quality control was our use of one single lot for each reagent for the entire study to eliminate possible batch differences due to different reagent lots.

Plates were run on a BioRad CFX96 with the following cycling parameters: 95^{o}C for 5 minutes; 2 cycles of 94^{o}C for 15 seconds, 49^{o}C for 15 seconds; 49 cycles of 15 seconds at 94^{o}C, 15 seconds at 62^{o}C, 15 seconds at 83^{o}C, 15 seconds at 60^{o}C with signal acquisition, 15 seconds at 94^{o}C, 20 seconds at 85^{o}C with signal acquisition. The 60^{o}C reads provided the the C_t values for the amplification of the telomere template (in early cycles when the albumin signal is still at baseline). The 85^{o}C reads provided the C_t values for the amplification of albumin template at which time the telomere template is fully melted. BioRad CFX Manager software version 3.0 automatically estimated the value for each T (telomere) and S (albumin single copy gene) using the standard curve.

Table 2: Primer Sequences for MMQPCR

Site	Primer Sequence
Telomere	telg: ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT telc: TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
Albumin	albu: CGGCGGCGGGCGGCGCGGGCTGGGCGGaaatgctgcacagaatccttg albd: GCCCGGCCCGCCGCGCCCGTCCCGCCGgaaaagcatggtcgcctgtt

Cell culture, Chromosome Harvest and Slide Preparation

Upon receiving the blood samples (in sodium heparin), leukocytes were isolated using Histopaque- 1077 (Sigma). Duplicate stimulated (using phytohemaglutinin (PHA)] lymphocyte cultures were established and harvested using standard procedures [RPMI 1640 media, supplemented with 15% Fetal bovine serum (FBS)]. Twenty minutes prior to harvesting, colcemid (final concentration of 0.1 μg/ml) was added in order to limit microtubule formation by inactivating spindle fiber formation, which arrested the cells in metaphase. Seventy-two hours after culture initiation, the cultures were harvested as previously described (Leach and Jackson Cook, 2001) using standard protocol techniques, including a 20-minute incubation in hypotonic solution (0.075 M KCl), a ten-minute incubation (room temperature) in fixative (3:1 methanol: acetic acid solution) and two additional washes in fixative. In order to reduce cytoplasm formation, which can compromise probe hybridization, slides were made using a Thermotron, which provided a constant temperature (23°C) and humidity (48 ±1°C). A phase contrast/bright field microscope was used to ensure that the quality of the slide preparation was optimal for the FISH analysis (i.e. enough metaphases present that lacked cytoplasm, had good morphology, and were well spread). The slides were then placed on a hot plate at 60°C for an hour, followed by aging at room temperature for 1-2 weeks. Alternatively, following hot plate incubation, room temperature aging was induced by soaking the slide in a 2xSSC solution for ten minutes prior to applying the FISH protocol.

Chromosome-Specific Telomere FISH

The telomere length of metaphase chromosomes was assessed using a telomere- specific FITC-labeled peptide nucleic acid (PNA) probe, following the manufacturer's protocol (DakoCytomation, Denmark). Briefly, target slides were soaked in cold fixative (3:1 methanol: acitic acid) for one hour. Following air-drying, the slides were rinsed in 1xTBS (Tris-Buffered Saline, PH 7.5) for two minutes, fixed in 3.7% formaldehyde in 1xTBS for two minutes, and then rinsed twice in 1xTBS (for five minutes each time). The slides were then immersed in a pre-treatment solution containing proteinase K for ten minutes and rinsed twice in 1xTBS for five minutes each. Following dehydration in an ethanol series (70%, 85% and 100%) for two minutes each, the slides were air-dried. After drying, 22 µl of FITC labeled PNA probe $(CCCTAA)_3$ was added to each full slide and a coverslip added. Following the co-denaturation of the probe and metaphase chromosomes in a thermocycler at 80°C for three minutes, the slides were hybridized in a dry hybridization chamber at room temperature for two hours. Unbound and excess probe was then removed by washing in a rinse solution (provided by the manufacturer) at room temperature for two minutes, as well as a second wash solution (provided by the manufacture) at 65°C for five minutes. After washing, the slides were dehydrated in a series of cold ethanol solutions (70%, 85% and 100%) for two minutes each. Finally, the slides were air-dried and counterstained with 24 µl of a 5:1 DAPI II /propidium iodide (Abott) solution.

Telomere Image Analysis

A total of ten metaphases for each individual were analyzed using software on an Applied Imaging Cytovision system (the Comparative Genomic Hybridization (CGH) function) as previously described (Leach et al., 2004). Briefly, for each metaphase analyzed, three images

were captured using a CCD camera. The first image was captured using a DAPI filter to allow for subsequent karyotyping and identification of the chromosomes based on their reverse DAPI banding pattern. The second image was captured using a FITC filter, which allowed for the visualization of the telomeric probe and is termed by the software as the "test image". The third image allowed for the visualization of the chromosomal body based on its propidium iodide stain and is termed a "reference image". Karyograms were prepared for each metaphase spread, followed by the designation of the centromere and central axis for each homolog. For each telomere, the software compared the fluorescence intensity from the "test image" to the fluorescence intensity of the propidium iodide stain in the chromosomal body from the "reference image" to generate a ratio profile for each chromosome. For each study participant the intensity values were averaged over the twenty homologs from the ten metaphase spreads scored. Overlapping chromosomes at the telomere region were omitted from the analysis. Representative images are shown in Figure 1.

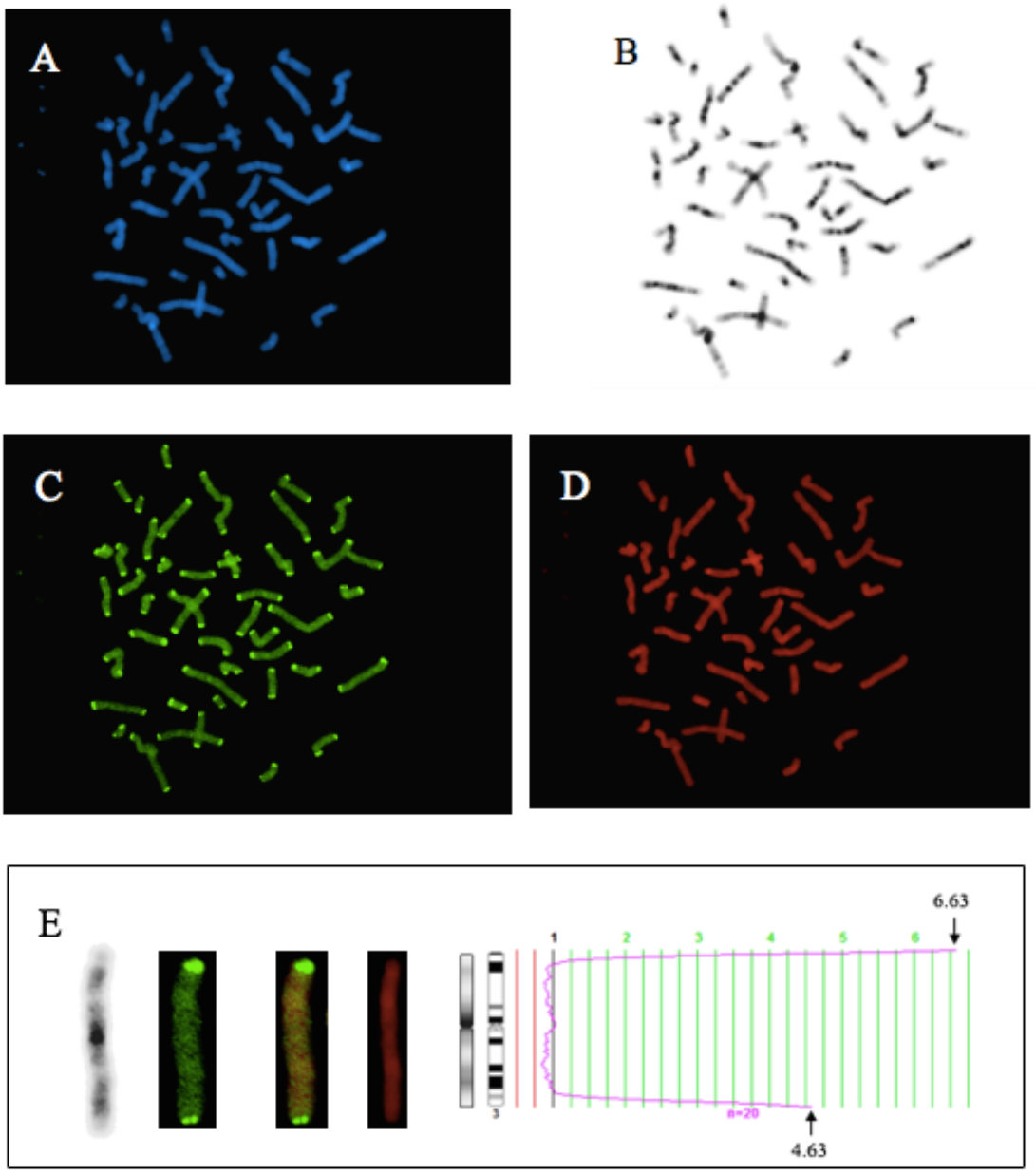

Figure 1: Representative images of chromosome-specific telomere FISH. For each individual, 10 metaphases are analyzed and for each metaphase 3 images are captured. (A) Shows a metaphase spread stained with DAPI. (B) The metaphase as it appears with reverse DAPI bands, allowing for chromosome recognition. (C) Shows a "test image" showing telomere signals at the end of each chromosome arm, captured using a FITC filter. (D) Represents the "reference image" showing the same metaphase with a propidium iodide stain. (E) A representative chromosome 3 from one participant as it appears in DAPI, FITC labeled, merged and PI image, respectively. Also, the ratio profile of this chromosome is shown. From this profile (and the visual image) one can see that the short arm telomere (top of chromosome) has a larger fluorescence intensity value (6.63) than the long arm telomere (bottom of chromosome) (value of 4.63).

Assessment of PNS

The following symptoms were measured by our collaborators at Virginia Commonwealth University School of Nursing according to the systems described:

- **Cognitive Dysfunction**

The CNS Vital Signs computerized neurocognitive testing system was used to measure cognitive dysfunction in our study participants (Gualtieri & Johnson, 2006). This system has been highly recommended for the use in longitudinal studies due to its high sensitivity to mild cognitive impairment. This system has the ability of excluding practice effects by randomly generating alternate test forms. Repeated measures of cognitive components with a concentration on memory and attention were achieved through a highly reliable and well-validated screening system (Gualtieri & Johnson, 2006).

- **Stress**

Percieved Stress Scale (PSS) is considered one of the widely used psychological instruments due to its well-documented reliability and validity (Cohen, Kamarck & Mermelstein, 1983). Participants rated how often they found their lives to be unpredictable, uncontrollable, and overloaded during the past month through a 10 item Percieved Stress Scale (PSS) (Cohen, Kamarck & Mermelstein, 1983). Each question has five possible Likert scale responses (never, almost never, sometimes, fairly often, and very often). Of the total responses on the PSS, 6 were “negative” responses and were given the following numerical value: never

= 0; almost never = 1; sometimes = 2; fairly often = 3; and very often = 4. The remaining 4 positive items in the PSS were recorded in a reversed format according to standard approaches (i.e. never = 4…very often = 0) (Innes et al., 2012, Abut et al., 2012). All 10 response values were then summed to calculate the participants' total perceived stress score, with higher scores indicating higher levels of perceived stress (Cohen, Kamarck & Mermelstein, 1983).

- **Depression and Anxiety**

The Hospital Anxiety and Depression Scale (HADS) was used to determine the presence and severity of depression and anxiety (Snaith, 2003). It is a questionnaire-based test where the study participant rated from 0-3 the severity of each symptom on 14 items over a period of 7 days. This assessment tool was selected for use in this study because it has been shown to have an excellent reliability and validity in patients with BC (Snaith, 2003).

- **Pain**

Pain was assessed using the Brief Pain Inventory (BPI), which is a self-reporting short form that is directed towards the assessment of pain severity and its impact on daily function in patients with pain from chronic diseases or cancer (Caraceni, 2001). Validity and reliability has been well established for these patients using this tool. Assessment areas include severity of pain, impact of pain on daily function, location of pain, pain medications and amount of pain relief in the past 24 hours or the past week. This test doesn't require a scoring algorithm; it relies on "worst pain" or the mean of the four severity items as a measure of pain severity, and the arithmetic mean of the seven interference items as a measure of pain interference (Caraceni, 2001).

- **Fatigue**

Fatigue was measured using the Brief Fatigue Inventory (BFI), which is a self-reporting test that is designed for patients with fatigue due to cancer and its treatment (Mendoza et al., 1999). This 9-item scale aids in the assessment of fatigue severity and the impact of fatigue on daily functioning in the past 24 hours. A global fatigue score can be obtained by averaging all the items on the BFI with severe fatigue being defined as a score of 7 or higher. This test has been used in clinical trials and has shown excellent reliability (Mendoza et al., 1999).

- **Sleep Disturbances**

The General Sleep Disturbance Scale (GSDS), as a means of obtaining self-reported sleep issues including problems initiating sleep, the number of awakenings, excessive daytime sleepiness and the use of medications to induce sleep over the past week (Lee, McEnany & Weekes, 1999). The 21 items in this tool are rated using a numeric rating scale ranging from 0 to 7. The total score for this scale ranges from 0 (no sleep disturbance) to 147 (extreme sleep disturbance) and is calculated by summing the 21 items (Lee, McEnany & Weekes, 1999).

Chromosome Instability Assessment

Chromosomal instability levels were quantified for each study participant using the cytokinesis-block micronucleus (CBMN) and cytome assay as previously described (Aboalela et al., 2015).

Statistical Analyses

Demographics, tumor characteristics and treatment variables in Tables 2-4 were computed using chi square tests for categorical data and a two sample t-test for continuous data when looking for differences in race (African Americans vs. Caucasian). A Pearson correlation was used to examine the correlation between age and telomere length, as well as between MN frequency and telomere length. The percent change in telomere intensity values for each chromosomal arm (p arm and q arm) from baseline to mid-chemo for each study participant was determined by the following formula:

$$\frac{(\text{Mid-chemo telomere intensity value} - \text{Baseline telomere intensity value})}{\text{Baseline telomere intensity value}} \times 100$$

A paired t-test was used to compare mean telomere intensity values for each chromosomal arm between baseline and mid-chemo.

Using the model building approach proposed by Hosmer and Lemeshow (2000), a mixed effects linear model (Brown & Prescot, 2006) was fit to determine the best subset of predictors of qPCR-based telomere length. In the first stage of the model building process, a base model was selected that represented the design of data collection and the timing of treatments (chemotherapy and radiation). Fixed effects included, visit (baseline, mid-chemo, six months following the initiation of chemo, 12 months and 24 months post-chemo), chemotherapy regimens, visit by chemotherapy interaction (chemotherapy was administered only during visit 2), radiation (Yes/No); visit by radiation interaction (radiation was only administered proximal to visit 3), and a random effect for study participants. In the second stage, each potential predictor

was fit individually with the base model and if the p-value was 0.25 or less, that predictor was used in the third stage. Potential predictors tested included demographic variables (age, race, and income), tumor characteristics (grade, stage, luminal A, luminal B, triple negative and HER2 positive status) and neoadjuvant status. In the third stage, all potential regressors ($p \leq 0.25$) were put into a multiple variable model. This initial model was further refined by sequentially removing variables from the model with the highest p-values (backward stepwise) until all remaining factors had a p-value of 0.05 or less. This model was considered the final prediction model. Least square means of significant predictor variables were tested using a Tukey test. To identify significant predictors of chromosome-specific telomere length, the previous base model was used with the exception of excluding radiation variable because chromosome-specific data was produced from only baseline and mid-chemo time points. In addition, instead of using a backward stepwise approach, each of the above mentioned potential predictors of chromosome-specific telomere length was fit individually and was considered a significant predictor when having a p-value of 0.05 or less. JMP Pro 12 statistical package was used for the mixed effect model and correlation analyses. R 3.2.0 software was used for the paired t-test.

Chapter 4

Results

A total of 77 women with early stage breast cancer (I – IIIA) were recruited for this study. One participant provided only a baseline specimen and then withdrew from the study citing a "lack of time/interest" as the reason for her withdrawal. Two women failed to provide specimens for all five time points and explained that they had a lack of time as their reason for non-compliance with the study protocol (96% retention rate). Only two women received cyclophosphamide, methotrexate and 5 fluorouracil (CMF) treatment. Therefore, due to the small sample size, this subset of participants was excluded from the analysis. Telomere length assessment was completed for a total of 72 participants of the 77 originally enrolled in the study. The majority of these study participants received TAC treatment (n= 39), followed by TC (n= 21), or TCH (n= 12).

Demographics

Demographics, breast tumor characterization and treatment information for the 72 women participating in this longitudinal study are shown in Tables 3-5. One woman in the African American cohort and one woman in the Caucasian cohort self-reported being of Hispanic or Latino ethnicity. Given the small number of the Hispanic sub-group, these women were included

in the African American and Caucasian sub-groups, respectively, but the Hispanic ethnic group was not analyzed separately. The age of the women fully participating in the study ranged from 23 to 71 years, with a median of 52 years. A significant difference in age was observed between the African American (n=22, mean = 46.6 years, standard error (s.e.) = 2.07 years) and Caucasian (n = 50, mean = 53.5 years, s.e. = 1.37 years) sub-groups ($p = 0.007$) (Table 3). Annual income also differed significantly between the two racial groups, with Caucasians having higher income than African Americans ($p < 0.0001$) (Table 3).

Tumor Characteristics

In accordance with this study design, only women with early stage breast cancer (stages I – IIIA) were included. There was no significant difference in the **grade** of the breast cancer tumors present in the African American compared to Caucasian women ($p = 0.197$) (Table 4). Similarly, no significant difference was observed for the **stage** of breast cancer between the two racial groups ($p = 0.055$) (Table 4). While there was no significant difference in the proportion of tumor sub-types, there was a trend toward a higher proportion of luminal A tumors and a lower proportion of HER2+ and triple negative tumors in the Caucasian population (60% luminal A tumors; 10% luminal B tumors; 6% HER2+; 24% triple negative tumors) compared to African Americans (36 % luminal A; 9% luminal B tumors; 18% HER2+; 36% triple negative tumors) (Table 4).

Treatment

The three main types of chemotherapy regimens administered to the study participants were categorized as 1) TAC, which included sequential administration of doxorubicin (Adriamycin),

cyclophosphamide (Cytoxan), and docetaxel (Taxotere); 2) TC, which included docetaxel (Taxotere) and cyclophosphamide (Cytoxan); and 3) TCH, which included docetaxel (Taxotere), Carboplatin (Paraplatin), and trastuzumab (Herceptin). No significant difference in neoaduvant/adjuvant status ($p = 0.123$), chemotherapy regimens ($p = 0.291$) or radiation treatment ($p = 0.229$) was observed between the racial groups (Table 5).

Table 3. Demographics for Study Participants Receiving Chemotherapy for Breast Cancer

	African American n= 22				**Caucasian** n= 50			
	TAC n=10 (45%)	**TC** n=6 (27%)	**TCH** n=6 (27%)	**AA Total** n= 22 (100%)	**TAC** n=29 (58%)	**TC** n=15 (30%)	**TCH** n=6 (12%)	**C. Total** n= 50 (100%)
Age	44.0 3.4	47.0 2.5	50.5 2.9		52.2 1.7	57.1 2.6	51.0 5.2	
Income								
Less than 30,000	5 (23%)	4 (18%)	3 (14%)	12 (55%)	6 (12%)	1 (2%)	0	7 (14%)
30,000 – 59,999	3 (14%)	2 (9%)	3 (14%)	8 (37%)	6 (12%)	1 (2%)	0	7 (14%)
60,000 – 89,999	1 (4%)	0	0	1 (4%)	6 (12%)	7 (14%)	3 (6%)	16 (32%)
90,000 +	1 (4%)	0	0	1 (4%)	11 (22%)	6 (12%)	3 (6%)	20 (40%)

- Number in parentheses is the percentage of participants in this category (%)
- For age, the mean (top line) and standard error (bottom line) are shown

Table 4. Breast Tumor Characteristics in the Study Participants

	African American n= 22	**Caucasian** n= 50
Luminal A		
Yes	8 (36.3%)	30 (60%)
No	14 (63.7%)	20 (40%)
Luminal B		
Yes	2 (9%)	5 (10%)
No	20 (91%)	45 (90%)
Triple negative		
Yes	8 (36%)	12 (24%)
No	14 (64%)	38 (76%)
HER2 positive		
Yes	4 (18%)	3 (6%)
N	18 (82%)	47 (94%)
Grade		
1	1 (4%)	4 (8%)
2	12 (55%)	16 (32%)
3	9 (41%)	30 (60%)

Stage		
I	5 (22%)	15 (30%)
IIA	11 (50%)	19 (38%)
IIB	6 (28%)	8 (16%)
IIIA	0	8 (16%)

- Number in parentheses is the percentage of participants in this category (%)

Table 5: Treatment Characteristics in the Study Participants

	African American n= 22	Caucasian n= 50
Neoadjuvant		
Yes	4 (18%)	3 (6%)
No	18 (82%)	47 (94%)
Herceptin		
Yes	6 (27.5%)	8 (16%)
No	16 (72.5 %)	42 (84%)
Radiation		
Yes	19 (87.5%)	37 (74%)
No	3 (12.5%)	13 (26%)

- Number in parentheses is the percentage of participants in this category (%)

Telomere Length Measured by MMqPCR

Of the total 72 women in this study, the quality of DNA from one patient did not meet quality control standards. Therefore, average genomic telomere lengths (which were assessed by the T/S ratio) were estimated for 71 study participants at all five time points using MMqPCR methodology. To determine if there was a potential association between telomere length at baseline and age, a Pearson's correlation was calculated. As expected, based on previous studies showing that telomere length is inversely correlated with age in the general population, a significant negative correlation between the participants' age and their average genomic telomere length was observed at baseline ($r = -0.37$, $p = 0.001$), as shown in Figure 2.

Given that telomere length has been associated with the frequency of spontaneous (and induced) chromosomal abnormalities in a variety of human cell types and health conditions (Leach et al., 2004), the T/S ratio values were compared to micronucleus/micronuclei (MN) frequencies (micronuclei are an indicator of chromosomal instability), at each time point using a Pearson correlation test. However, no significant associations were observed between mean telomere length and MN frequency [visit 1 T/S ratio and visit 1 MN frequency ($r = -0.084$, $p = 0.491$); visit 2 T/S ratio and visit 2 MN frequency ($r = -0.054$; $p = 0.659$); visit 3 T/S ratio and visit 3 MN/ frequency ($r = -0.016$, $p = 0.896$); visit 4 T/S ratio and visit 4 M/N frequency ($r = -0.047$, $p = 0.708$); visit 5 T/S ratio and visit 5 MN frequency ($r = -0.203$, $p = 0.197$)].

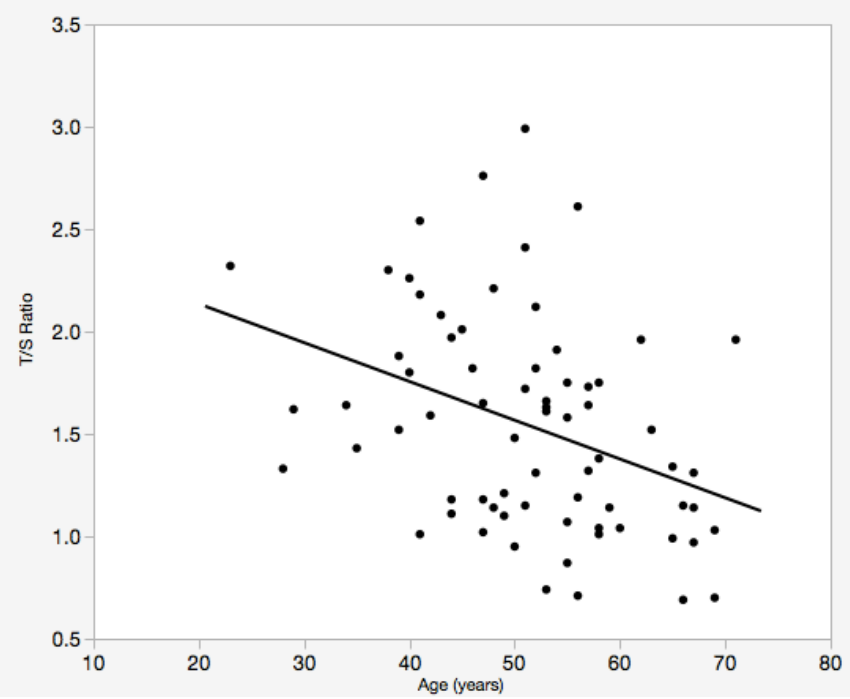

Figure 2: Association between age and average genomic telomere length at baseline. Each circle (•) represents a study subject. Average genomic telomere length at baseline was negatively correlated with age (r = -0.37, p = 0.001).

In addition to the assessment of MN frequency, we also investigated the relationship between telomere length change and other treatment, demographic variables and PNS by fitting the data into a mixed effects linear regression model (Table 6). Potential predictive variables evaluated included visit, age, race, income, tumor grade and stage, receiving Herceptin, having neoadjuvant treatment, tumor characterization as luminal A, luminal B, triple negative or HER2 positive and PNS including anxiety, depression, pain, fatigue, sleep disturbance and stress. The final optimized model identified age ($p = 0.004$), race ($p = 0.019$) and the type of chemotherapy treatment ($p = 0.044$) as having a significant predictive association with mean telomere length (Table 6). For the racial groups, the average genomic telomere lengths were higher in African American participants (mean = 1.661; s.e. = 0.087) compared to Caucasian participants (mean = 1.420; s.e.= 0.061) (Table 6). The study visit time was not found to be a significant predictor of telomere length ($p = 0.666$). However, the shortest telomere lengths tended to be observed at visit 3 ($p = 0.41$) (where a subset of patients were on radiation treatment), with values by 12 months and 24 months following the initiation of chemotherapy tending to be comparable to values at baseline (Figure 3). While visit alone was not predictive of mean telomere length, the type of chemotherapy used in treatment was found to be a significant predictor of telomere length ($p = 0.044$), with the subset of women receiving TAC treatment having significantly higher mean telomere lengths (mean = 1.681; s.e. = 0.074) than the women receiving TCH (mean = 1.366; s.e. = 0.103) chemotherapy regimens ($p= 0.036$) (table 5). However, women receiving TC treatment were not significantly different from TCH ($p= 0.272$) or TAC ($p= 0.535$) groups. Other variables screened were found to have no significant prediction of telomere length (Table 6).

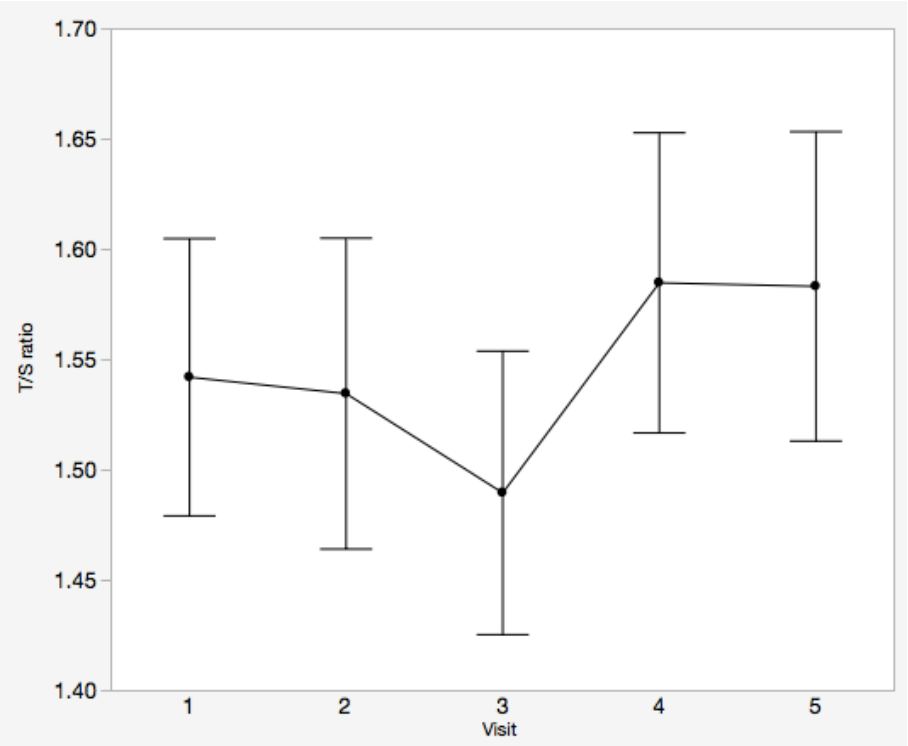

Figure 3: Vlues for MMqPCR based telomere length over time. Average genomic telomere lengths are shown at baseline (visit 1) (mean= 1.542, s.e.= 0.062), mid-chemo (visit 2) (mean= 1.534, s.e.= 0.070), visit 3 (mean= 1.489, s.e.= 0.064). visit 4 (mean= 1.584, s.e.= 0.068) and visit 5 (mean= 1.583, s.e.= 0.070).

Table 6: Mixed Effects Linear Model Fitting Assessment of Predicative Associations of Variables with qPCR Telomere Length

	Base Model[1]	Final Model[2]		
Variable	**p-value**	**Least Squares Mean**	**Std. Error**	**p-value**
Visit	0.666			
Age*				0.004
Race*				0.019
African Americans		1.661	0.087	
Caucasians		1.420	0.061	
Income	0.083			
Chemotherapy*				0.044
TAC		1.681	0.074	
TC		1.575	0.088	
TCH		1.366	0.103	
Chemotherapy by visit	0.259			
Radiation	0.641			
Radiation by visit	0.271			
Stage	0.148			
Grade	0.954			
Herceptin Yes/No	0.857			
Neoadjuvant	0.506			
Luminal A	0.891			
Luminal B	0.529			
Triple negative	0.997			

HER2 positive	0.350
Anxiety	0.461
Depression	0.633
Pain	0.946
Fatigue	0.759
Sleep disturbance	0.825
Stress	0.760

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[1] The base model, which was determined by the study design, was: telomere length = Visit + Chemotherapy (3 types) + Radiation therapy +Visit by chemotherapy[3] + Visit by Radiation therapy[3] with the study subject being the random effect

[2] The final model reflects variables that remained significantly associated with telomere length after stepwise removals

[3] The visit by chemotherapy and visit by radiation therapy variables allowed these values to differ across time points

* Variables significantly predicative of telomere length

Telomere Length Quantified Using Chromosome-Specific FISH

Of the 72 women included in this study, blood specimens obtained from 22 patients did not yield metaphases from either baseline and/or the mid-chemo specimen. Therefore, chromosome-specific telomere lengths were compared for 50 subjects. In order to determine if there was a change in the telomere intensity value of each chromosomal arm (p arm and q arm) from baseline (visit 1) to mid-chemo (visit 2) within and across study participants, the percent change in telomere intensity values was calculated. Negative values reflected a decrease in telomere length at mid- chemo compared to baseline; a zero value reflected no change in telomere length; and a positive value reflected an increase in telomere length at the mid-chemo compared to baseline time points. The percent change values for telomeres on each chromosomal arm (short arm and long arm) and each study participant is graphically represented using a heat map (Figure 4). Interestingly, while we observed that the majority of telomeres showed attrition, a small subset of telomeres showed increases in length as shown in Figure 5.

In addition to calculating percent changes, we compared telomere fluorescent intensities of the short and long arm of the 23 chromosomes (1-22 and X) between baseline and mid-chemo using a paired t-test. Significantly shortened telomeres were found on 1p ($p = 0.022$), 5q ($p = 0.041$), 7q ($p = 0.025$), 9q ($p = 0.045$), 18q ($p = 0.002$), 20p ($p = 0.020$), 21q ($p = 0.040$) and 22p ($p = 0.025$), with marginal significance for decreases of telomere length being observed for 6p, 6q, 7p, 8p, 9p, 10q, 13p, 13q, 14q, 17q, 18p, and 22q (Table 7).

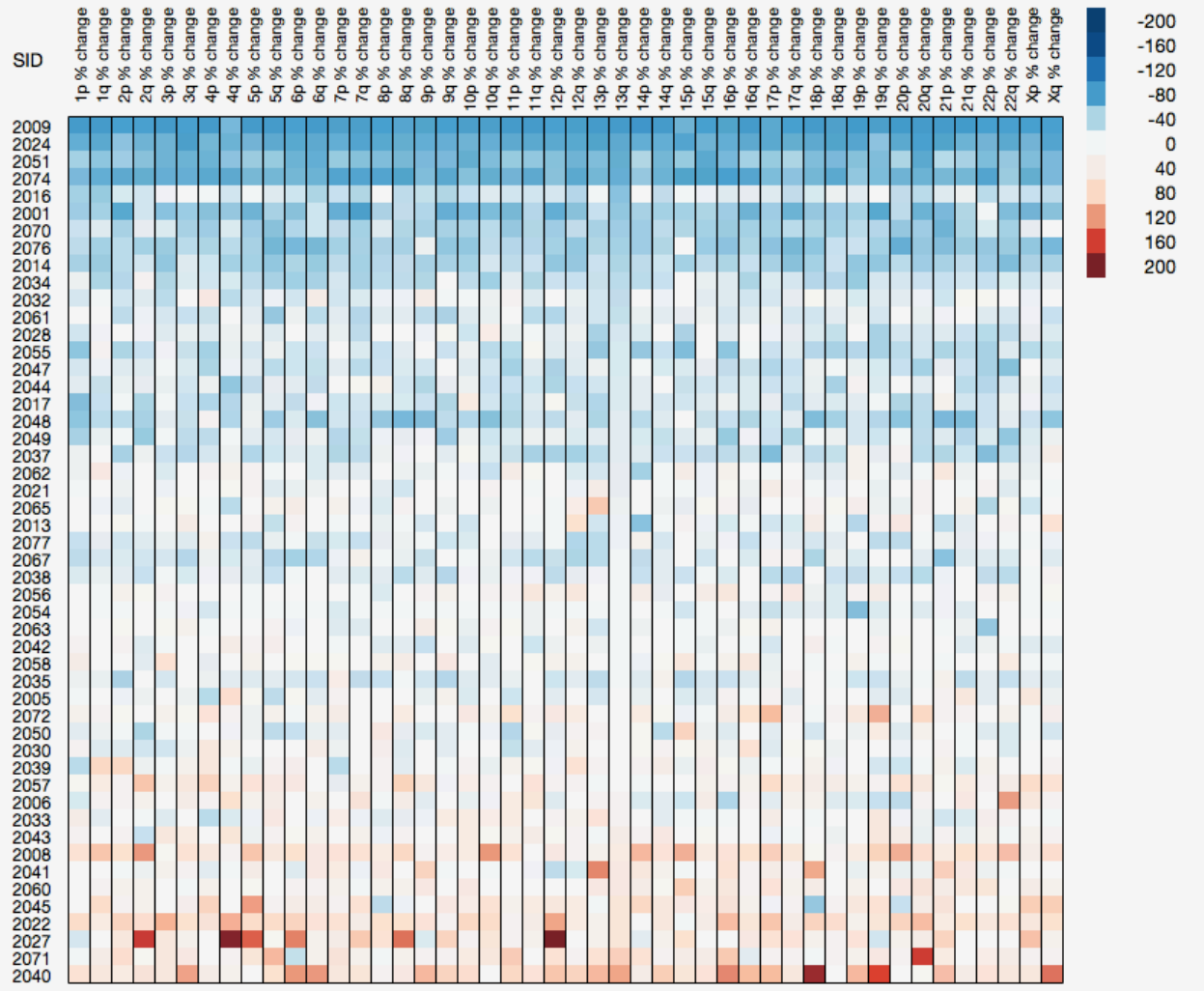

Figure 4: Heat map representation of percent changes in telomere intensity values from baseline to mid-chemo. Each column represents a chromosomal arm and each row represents a study participant [Study Identifier (SID)]. The legend shows the percent change, with decreases in telomere length represented by negative values (shown in blue tones), whereas increases in telomere length are represented by positive values (shown in red tones).

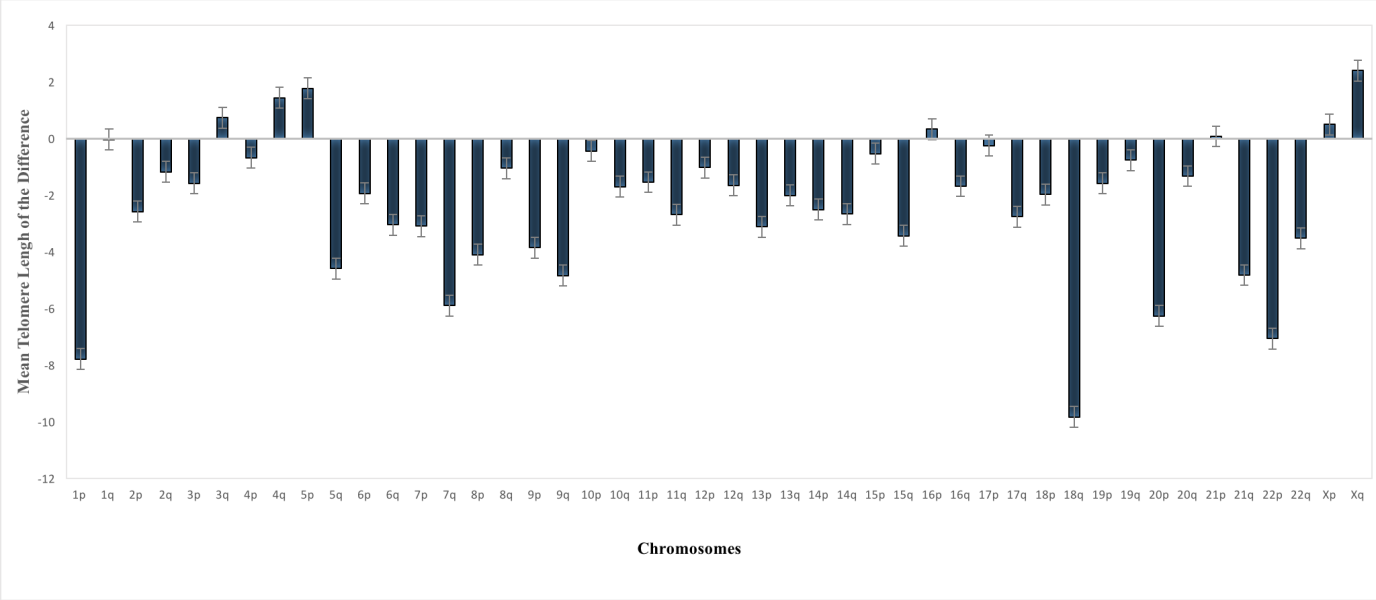

Figure 5: Mean of chromosome-specific telomere lengths in all study participants (n = 50). The average of the difference in telomere length from baseline to mid-chemo is shown for each chromosomal arm.

Table 7: A Comparison of Chromosome Intensity Values Between Baseline and Mid-Chemo Specimens in 50 Women with Early Stage Breast Cancer

Chromosome	Short Arm						Long Arm					
	Baseline		Mid-Chemo				Baseline		Mid-Chemo			
	Mean	SD	Mean	SD	Mean of the difference	p-value	Mean	SD	Mean	SD	Mean of the difference	p-value
1	4.085	1.702	3.556	1.767	-0.529	**0.022**	5.292	2.058	4.955	2.228	-0.337	0.272
2	5.291	2.148	4.787	2.184	-0.504	0.102	4.084	1.732	3.718	1.793	-0.366	0.155
3	6.054	2.128	5.579	2.233	-0.475	0.144	5.148	2.238	4.714	2.108	-0.434	0.158
4	4.349	1.903	3.947	1.856	-0.402	0.160	5.588	2.155	5.204	2.173	-0.384	0.195
5	5.107	2.074	4.749	2.072	-0.358	0.241	4.811	2.135	4.186	2.029	-0.625	**0.041**
6	5.452	2.171	4.866	2.118	-0.586	0.084	5.180	2.119	4.614	2.134	-0.566	0.069
7	4.419	1.868	3.935	1.763	-0.484	0.058	5.182	2.107	4.449	2.058	-0.733	**0.025**
8	5.222	2.017	4.663	1.970	-0.559	0.054	4.396	1.993	4.052	2.105	-0.344	0.198
9	5.429	2.080	4.879	2.142	-0.550	0.062	3.781	1.768	3.322	1.731	-0.459	**0.045**
10	5.326	2.117	4.967	2.188	-0.359	0.228	4.621	2.004	4.170	1.935	-0.451	0.093
11	4.587	1.897	4.204	1.994	-0.383	0.173	5.123	2.110	4.693	2.201	-0.430	0.134
12	4.636	1.909	4.194	2.093	-0.442	0.145	4.659	1.958	4.216	2.009	-0.443	0.142
13	4.858	1.963	4.340	1.999	-0.518	0.064	5.331	2.202	4.809	2.164	-0.522	0.090
14	4.827	2.069	4.432	2.256	-0.395	0.165	4.754	2.135	4.275	1.952	-0.479	0.069
15	4.544	1.969	4.208	1.976	-0.336	0.218	5.193	2.117	4.737	2.170	-0.456	0.111
16	3.920	1.796	3.484	1.590	-0.436	0.119	4.305	1.869	3.938	1.955	-0.367	0.167
17	4.106	1.765	3.717	1.714	-0.389	0.150	4.118	1.815	3.714	1.782	-0.404	0.099
18	5.188	2.141	4.596	2.056	-0.592	0.051	5.496	2.098	4.620	1.877	-0.876	**0.002**
19	3.997	1.791	3.631	1.686	-0.366	0.149	4.580	1.887	4.135	1.901	-0.445	0.135
20	4.979	2.060	4.282	1.977	-0.697	**0.020**	4.187	1.805	3.743	1.949	-0.444	0.134
21	5.060	2.124	4.543	1.955	-0.517	0.108	4.413	1.867	3.897	1.740	-0.516	**0.040**
22	4.902	2.016	4.239	1.949	-0.663	**0.025**	4.058	1.795	3.610	1.827	-0.448	0.090
X	5.240	2.103	4.893	2.149	-0.347	0.236	4.850	2.075	4.589	2.199	-0.261	0.385

In agreement with the paired t-test results, a mixed effects model adjusted for age showed that there is a significant shortening in chromosome-specific telomere length from baseline to mid-chemo on chromosomal arms 1p ($p = 0.022$), 5q ($p = 0.041$), 7q ($p = 0.025$), 9q ($p = 0.045$), 18q ($p = 0.002$), 20p ($p = 0.020$), 21q ($p = 0.040$) and 22p ($p = 0.025$) (Table 8).

To further assess the association between chemotherapy treatment on telomere lengths, a fixed effects model, adjusted for age, was also fit to the chromosome-specific data. The type of chemotherapy treatment was observed to be predictive of telomere lengths on 32 of the 46 chromosomal arms (Table 9), with participants receiving TAC chemotherapy treatment have numerically higher mean telomere lengths on those chromosomes compared to study participants receiving TC or TCH treatment (Table 9).

To determine if there was an association between telomere length and PNS we included "total" values for the symptoms of anxiety, depression, pain, fatigue, sleep disturbance and stress into the mixed effect model, adjusted for age, to assess their predictive potential of chromosome-specific telomere length. Among all the PNS tested, pain (but no other symptoms) was found to be a significant negative predictor of telomere lengths (higher pain; shorter telomeres) for 5q ($p = 0.040$), 8p ($p = 0.047$), 13p ($p = 0.019$), 20p ($p = 0.036$), 22p ($p = 0.035$), Xp ($p = 0.014$), Xq ($p = 0.039$) (Table 10).

Table 8: Mixed Effects Linear Model Fitting Assessment of Visit as a Significant Predicative variable of Chromosome-Specific Telomere Length

Chromosome Arm	Least Square Mean at Baseline	Least Square Mean at Mid-Chemo	Std. Error	p-value
1p	3.632	3.103	0.264	0.022
5q	4.428	3.803	0.330	0.041
7q	4.757	4.024	0.331	0.025
9q	3.376	2.917	0.217	0.045
18q	5.078	4.204	0.311	0.002
20p	4.635	3.938	0.320	0.020
21q	3.954	3.438	0.274	0.040
22p	4.499	3.835	0.305	0.025

Table 9: Mixed Effects Linear Regression Model Fitting Assessment of Predictive Association of Chemotherapy Type with Chromosome-Specific Telomere Length

Chromosome Arm	TAC Least Square Mean	TC Least Square Mean	TCH Least Square Mean	P-value
1p	**4.346 (0.251)**	**2.944 (0.451)**	**2.813 (0.501)**	**0.004**
1q	**5.642 (0.314)**	**4.016 (0.564)**	**4.433 (0.626)**	**0.028**
2p	**5.602 (0.315)**	**3.929 (0.565)**	**4.174 (0.628)**	**0.017**
2q	4.247 (0.263)	3.125 (0.473)	3.486 (0.526)	0.094
3p	6.252 (0.319)	4.671 (0.574)	5.508 (0.637)	0.059
3q	**5.488 (0.317)**	**3.853 (0.570)**	**4.051 (0.634)**	**0.020**
4p	4.475 (0.280)	3.671 (0.502)	3.436 (0.558)	0.157
4q	5.876 (0.326)	4.425 (0.586)	4.689 (0.651)	0.059
5p	**5.451 (0.295)**	**3.696 (0.531)**	**4.376 (0.589)**	**0.014**
5q	4.590 (0.309)	3.682 (0.556)	3.714 (0.618)	0.064
6p	**5.698 (0.298)**	**4.028 (0.536)**	**4.417 (0.596)**	**0.015**
6q	**5.383 (0.312)**	**3.835 (0.561)**	**4.279 (0.623)**	**0.039**
7p	**4.598 (0.269)**	**3.465 (0.484)**	**3.383 (0.538)**	**0.043**
7q	**5.335 (0.305)**	**3.673 (0.549)**	**4.163 (0.609)**	**0.022**
8p	**5.453 (0.288)**	**3.821(0.518)**	**4.301 (0.575)**	**0.016**
8q	4.685 (0.312)	3.252 (0.561)	3.593 (0.623)	0.056
9p	**5.664 (0.314)**	**4.201 (0.564)**	**4.304 (0.627)**	**0.034**
9q	**4.037 (0.259)**	**2.581 (0.465)**	**2.821 (0.517)**	**0.012**
10p	**5.729 (0.313)**	**4.114 (0.562)**	**4.107 (0.624)**	**0.013**
10q	4.821 (0.299)	3.726 (0.537)	3.531 (0.597)	0.070
11p	4.791 (0.292)	3.639 (0.524)	3.758 (0.582)	0.090
11q	**5.413 (0.327)**	**4.152 (0.587)**	**3.837 (0.653)**	**0.045**
12p	**4.890 (0.287)**	**3.289 (0.515)**	**3.918 (0.572)**	**0.023**
12q	4.838 (0.289)	3.674 (0.520)	3.791 (0.578)	0.083
13p	**5.093 (0.289)**	**3.422 (0.519)**	**4.096 (0.576)**	**0.018**
13q	**5.599 (0.323)**	**4.146 (0.580)**	**4.111 (0.645)**	**0.033**
14p	**5.221 (0.314)**	**3.349 (0.565)**	**3.864 (0.628)**	**0.010**

14q	**5.000 (0.312)**	**3.487 (0.561)**	**3.856 (0.623)**	**0.041**
15p	**4.885 (0.285)**	**3.131 (0.512)**	**3.896 (0.569)**	**0.012**
15q	**5.535 (0.318)**	**3.823 (0.571)**	**4.112 (0.634)**	**0.017**
16p	**4.064 (0.237)**	**2.993 (0.426)**	**3.137 (0.473)**	**0.049**
16q	**4.614 (0.281)**	**3.126 (0.504)**	**3.398 (0.560)**	**0.020**
17p	4.282 (0.250)	3.083 (0.449)	4.464 (0.499)	0.051
17q	4.265 (0.276)	3.126 (0.496)	3.507 (0.551)	0.110
18p	5.224 (0.322)	4.218 (0.578)	4.404 (0.643)	0.238
18q	**5.556 (0.292)**	**4.058 (0.526)**	**4.310 (0.584)**	**0.024**
19p	**4.281 (0.248)**	**2.280 (0.445)**	**3.185 (0.495)**	**0.010**
19q	**4.852 (0.261)**	**3.232 (0.469)**	**3.786 (0.522)**	**0.009**
20p	5.054 (0.300)	3.684 (0.539)	4.122 (0.599)	0.067
20q	**4.430 (0.262)**	**2.917 (0.470)**	**3.414 (0.522)**	**0.015**
21p	**5.356 (0.278)**	**3.474 (0.500)**	**4.242 (0.555)**	**0.005**
21q	**4.700 (0.257)**	**3.121 (0.462)**	**3.267 (0.513)**	**0.004**
22p	**5.068 (0.282)**	**3.438 (0.506)**	**3.995 (0.562)**	**0.016**
22q	**4.269 (0.264)**	**3.086 (0.475)**	**3.028 (0.527)**	**0.031**
Xp	5.518 (0.322)	4.320 (0.579)	4.190 (0.643)	0.077
Xq	**5.237 (0.316)**	**3.834 (0.567)**	**3.756 (0.630)**	**0.032**

* The standard error is shown in parenthesis

* Bold rows indicate significance ($p \leq 0.05$)

Table 10: Pain as a Significant Negative Predictor of Telomere Length

	Parameter Estimate	Standard Error	p-value
Chromosome			
1p	-0.165	0.099	0.103
1q	-0.196	0.124	0.121
2p	-0.127	0.126	0.319
2q	-0.146	0.105	0.170
3p	-0.185	0.127	0.151
3q	-0.182	0.126	0.156
4p	-0.131	0.112	0.248
4q	-0.185	0.130	0.160
5p	-0.188	0.117	0.115
5q	**-0.120**	**0.120**	**0.040**
6p	-0.131	0.120	0.279
6q	-0.222	0.122	0.076
7p	-0.197	0.105	0.069
7q	-0.119	0.123	0.335
8p	**-0.229**	**0.112**	**0.047**
8q	-0.195	0.123	0.122
9p	-0.242	0.122	0.054
9q	-0.164	0.102	0.116
10p	-0.199	0.124	0.114
10q	-0.202	0.118	0.093
11p	-0.177	0.116	0.133
11q	-0.203	0.129	0.123
12p	-0.175	0.113	0.130
12q	-0.215	0.113	0.064
13p	**-0.267**	**0.110**	**0.019**
13q	-0.197	0.128	0.131

14p	-0.158	0.126	0.215
14q	-0.189	0.124	0.133
15p	-0.186	0.112	0.106
15q	-0.216	0.125	0.090
16p	-0.168	0.093	0.077
16q	-0.204	0.110	0.070
17p	-0.164	0.099	0.103
17q	-0.212	0.108	0.055
18p	-0.252	0.125	0.051
18q	-0.181	0.116	0.124
19p	-0.145	0.098	0.148
19q	-0.187	0.102	0.075
20p	**-0.251**	**0.116**	**0.036**
20q	-0.126	0.105	0.235
21p	-0.185	0.110	0.099
21q	-0.139	0.102	0.180
22p	**-0.237**	**0.109**	**0.035**
22q	-0.189	0.103	0.075
Xp	**-0.311**	**0.122**	**0.014**
Xq	**-0.260**	**0.122**	**0.039**

* Bold rows indicate significance ($p \leq 0.05$)

Chapter 5

Discussion

In this study we sought to measure telomere length in lymphocytes obtained from patients who received chemotherapy for breast cancer using two different, but complementary, telomere length assays. The first was a qPCR-based assay, which is considered a high throughput assay with fast turn-around time that allows for the estimation of average genomic telomere length. The second assay used was a chromosome-specific PNA-FISH assay, which allows for the quantification of telomere lengths on each chromosomal arm, as well as the mean genomic telomere length. The use of both assays provided a confirmatory opportunity for the results discussed herein.

Consistent with the results of other investigators (Svenson et al., 2008, Wang et al., 2011), baseline telomere lengths of the women in our study showed a significant inverse correlation with age (older women had shorter telomeres). Furthermore, age was found to be a significant predictor of telomere length in our study cohort. Telomere attrition is one of the several cellular and molecular hallmarks contributing to the aging process and arises as a result of the end replication problem (reviewed in Aubert & Lansdorp, 2008, Lopez-Otin et al., 2016). It is thought that environmental and life-style related factors, such as smoking, exposure to pollution, lower physical activity, psychological stress, and unhealthy diet also contribute to accelerating telomere shortening and the ageing process via increased levels of inflammation and oxidative burden (von Zglinicki, 2000, Cherkas et al., 2008, Cassidy et al., 2010, Barrett et al., 2015, reviewed in Zhang et al., 2016). Furthermore, the sequence motif in human telomeres

(TTAGGG), which have a triple guanine structure, is thought to make them particularly susceptible to oxidative damage, with the repair of this oxidative damage being relatively inefficient (Kawanishi, Hiraku & Oikawa, 2001, von Zglinicki, 2002).

Another demographic variable that was observed to be a significant predictor of mean telomere length in our study cohort was race, with African Americans having longer mean telomere lengths than Caucasians. This observation has been reported in other studies investigating the relationship of race with telomere length in families with high and low risk of cardiovascular disease (Hunt et al., 2008), as well as healthy individuals (Lynch et al., 2016). Interestingly, in a case-control study on the association between leukocyte telomere length and breast cancer risk, Zheng, et al. (2010) reported higher mean telomere lengths in African Americans compared with Caucasians in newly diagnosed, pretreatment cases with breast cancer, but not in matching controls (Zheng et al., 2010). The mechanism(s) underlying this racial difference in telomere length is/are not well understood. One reason might be that healthy African Americans were found to have lower oxidative damage levels and lower plasma levels of antioxidants than Caucasians, suggesting that there might be race-related differences in the cellular processes involved in oxidative-related DNA damage and repair (Huang, Helzlsouer, &Appel, 2000, Watters et al., 2007, Watters, Satia, & Kupper, 2008). Alternatively, although the mixed effect model used in this study allowed adjusting for age, the inter-relatedness of human epidemiology variables increases the possibility for the presence of an effect from other confounding factors. Thus, one cannot fully rule out the possibility that the age distribution in our study cohort may have contributed to this association with race since the African American participants were significantly younger than the Caucasians (Aboalela, et al., 2015). Moreover, our model was not adjusted for other potential confounding factors that have been demonstrated

to be associated with higher oxidative stress or short telomere length, including (but not limited to) body mass index (BMI), smoking status, alcohol consumption, physical activity and the intake of antioxidants (Watters, Satia, & Kupper, 2008, Muezzinler, Zaineddin, & Brenner, 2013, Muezzinler et al., 2015, Muezzinler et al., 2016).

The main aim of this study was to test our hypothesis that chemotherapy contributes to chromatin breakage within the telomere, thereby leading to telomere shortening in women with early stage breast cancer. Using the data from the qPCR-based method, we did not observe a significant change in mean telomere lengths across the 5 time points in this 2-year longitudinal study. Although telomere length in epidemiological studies is most often evaluated as a single mean genomic measure, investigators have shown that there is heterogeneity between chromosomes (and between individuals) for telomere length, as well as variability between telomeric arms (short arm compared to long arm) within a chromosome (Lansdorp et al., 1996, Hemann et al., 2001, Gilson & Londono-Vallejo, 2007, Xu et al., 2013). Moreover, the results of a number of reports have suggested that telomere shortening does not occur at an equal rate among all chromosomal arms and that chromosomal arms with the shortest telomeres are most frequently involved in end-to-end fusions and genomic instability (Hemann et al., 2001, Soler et al., 2005). However, the qPCR-based mean telomere length assay cannot fully detect chromosome-specific heterogeneity (e.g. presence of relatively long telomeres could mask the presence of a sub-set of short telomeres present on specific chromosomal arms), nor can it capture information about telomeres that are extremely short/missing. Therefore, given the above mentioned limitations of the qPCR methodology, we elected to also measure chromosome-specific telomere lengths.

By exploiting the strength of our longitudinal study design for this chromosome-specific comparison, we were able to quantify **changes** in telomere length within a study subject (thereby reducing the impact of person-to-person variations in telomere lengths). The results of this analysis showed significant telomere attrition for a subset of 8 different chromosomal arms; specifically, 1p, 5q, 7q, 9q, 18q, 20p, 21q, and 22p. There are very few investigators who have evaluated chromosome-specific telomere lengths as they relate to cancer, with these few studies being concentrated on the relationship between chromosome-specific length and the risk of acquiring breast cancer or esophageal cancer (Table 11) (Zheng et al., 2009, Xing et al., 2009, Zheng et al., 2011). Therefore, our observation is considered an initiating step for investigating possible mechanisms responsible for these chromosomes to be preferably shortened during/after treatment for breast cancer.

Two main factors that have been conjectured to drive telomere length shortening, beside the end replication problem, are inflammation and oxidative DNA damage (von Zglinicki, 2002). Our observation that the type of chemotherapy received was predictive of telomere length is consistent with these canonical influences. There are several potential mechanisms that could explain our observed association between chemotherapy and telomere length. Genotoxic drugs may have an intrinsic role in telomere shortening given that telomeres have been shown to be particularly susceptible to oxidative damage and since repair of this damage is relatively inefficient in telomeres (von Zglinicki, 2000, von Zglinicki 2002, Shen et al., 2009, Fumagalli et al., 2012).

In addition to exerting their therapeutic effect through direct DNA damage, chemotherapeutic drugs that are frequently used in treating breast cancer have an indirect negative impact on

telomeres by affecting members of the shelterin complex. Among the six shelterin components, TRF2 and POT1 were found to be the most important in ensuring chromosome end protection by preventing ATM and ATR dependent checkpoint activation (van Steensel, Smogorzewska, & de Lange, 1999, Karlseder et al., 1999, Denchi & de Lange, 2007, Guo et al., 2007). Moreover, doxorubicin has been found to reduce the expression of POT1 and TPP1, which in turn could alter the telomere structure and compromise its stability, thereby promoting telomere shortening (Li et al., 2012, Kato et al., 2013).

Another class of chemotherapeutic drugs frequently used in treating breast cancer is Taxanes, which act through mitotic inhibition (Janssen & Medema, 2011). Although the exact mechanism for the induction of cell death upon mitotic inhibition is unknown, it is proposed that prolonged mitotic inhibition, in vitro, has the ability to dissociate TRF2 from telomeres and activate a DNA damage response, thereby providing a mechanism for telomere shortening through telomere dysfunction. (Hayashi et al., 2012, Lu et al., 2013).

In addition to showing that overall chemotherapy was predictive of telomere length, we also showed that there were differential effects based on the type of chemotherapy used in treatment, with telomere lengths of women receiving TAC regimens being significantly different from those receiving TCH regimens. Adriamycin (also known as doxorubicin) is a member of the anthracycline class of chemotherapeutic agents. Anthracyclines are thought to shorten telomeres through direct DNA damage by free radical formation (von Zglinicki, 2000, Quiles et al., 2002, Thorn et al., 2011). Another proposed mechanism of action for anthracyclines is the inhibition of DNA repair enzymes, particularly topoisomerase II, which may contribute to telomere

shortening through further accumulation of DNA damage (Table 12) (Elmore et al., 2002, Maccormick, 2006, Thorn et al., 2011, Buttiglieri, et al., 2011).

While a subset of women receiving TAC had shortened telomeres, we also observed the unexpected result that overall patients on the TAC regimen had longer mean telomere lengths when compared to patients undertaking TCH regimens. Similarly, the model used for the analysis of the chromosome-specific telomere data showed that patients on the TAC regimen had numerically higher telomere lengths than patients undertaking TC or TCH. One of the key components to the TAC regimen that is not present in the TCH regimen is the presence (or absence) of Adriamycin, which has been shown to have several cytotoxic effects and has been associated with telomere shortening, as noted above. When coupling the knowledge of the cellular effects of Adriamycin with our study results, they initially seemed to be paradoxical. However, a plausible explanation could be that ALT is activated as a rescue mechanism of the extensive DNA damage caused by TAC. From experiments on immortalized human cell lines that utilize the ALT mechanism for telomere length maintenance, it is hypothesized that ALT involves homologous-recombination (HR) mediated DNA copying of telomeric DNA (Dunham et al., 2000, Conomos, Picket & Reddel, 2013). One can speculate that telomeres become uncapped due to excessive shortening; presumably because they are no longer able to bind sufficient shelterin proteins (Denchi & de Lange, 2007, Sfeir & de Lange, 2012). Given the role of TRF2 and POT1 in suppressing HR-mediated DNA damage repair (Rai et al., 2016), it is reasonable to speculate that depletion of both proteins as an effect of Adriamycin would allow the activation of ALT through HR-mediated DNA repair.

One of the fundamental questions addressed in this study was the association between telomere length and PNS in patients treated for early stage BC. While no statistically significant associations between PNS and overall mean telomere length (using a qPCR method) were observed, our chromosome-specific telomere length data indicated that pain is a significant negative predictor of the length of the telomeres localized to 5q, 8p, 13p, 20p, 22p, Xp and Xq. Standard medical interventions of breast cancer (i.e., surgery, chemotherapy and/or radiotherapy) have pain as a common consequence (Starkweather, Lyon, & Schubert, 2011, Ferreira et al., 2015). The relationship between proinflammatory cytokines, including (but not limited to) CRP; TNFα; IL-2; IL-6; IL-8; IL-10, and pain has been established in many clinical conditions associated with inflammation, with higher levels of these inflammation markers being associated with greater pain intensity/sensitivity (reviewed in DeVon et al., 2014). Excessive telomere shortening arising as a result of oxidative stress mediated by increased inflammatory activity from the cancer itself, or chemotherapy, may lead to increased replicative senescence. Given that lymphocytes with shortened telomeres and senescent cells hypersecrete proinflammatory cytokines, particularly IL-6 (Efross, 2009), accelerated telomere attrition may play a role in the development of pain in our study cohort by contributing to the increased levels of IL-6. Furthermore, since chronic pain represents a physiological stressor, the associated increase in pro-inflammatory cytokines, upon activation of the stress pathway, may also be considered as a potential pathway for increased telomere shortening, thereby creating a cycle of telomere shortening from high levels of inflammation and increased pain sensation from increased telomere attrition-mediated cellular senescence.

Given that IL-6 molecules are not retained for a long period of time, the above proposed mechanisms may be an initiating step, with telomere biology playing a role in the persistence of

pain in patients with breast cancer into survivorship. However, the nature of the association between pain and telomere length is not clear. It is possible that this relationship is merely correlative, with the telomere length serving as a "canary in a coal mine" type observation that enables one to document that a biologically relevant change has occurred. Alternatively, it has been speculated that telomere length shortening could initiate epigenetic changes in genes that, in turn, contribute to acquired phenotypes. These epigenetic changes could arise as a result of the telomere position effect (TPE), which involves the spreading of a heterochromatic conformation of the DNA juxtaposed to the telomere to silence subtelomeric genes, with this phenomenon functioning in relation to telomere length. In a study of a complex-age associated disease, facioscapulohumeral muscular dystrophy (FSHD), TPE was shown to extend at least 100 kb into the subtelomeric region of chromosome 4q where the gene that is linked to the disease is up-regulated by an effect of telomere shortening (Stadler et al., 2013). While the biological cascade of events is not known, one could speculate that a change in the chromatin structure of the subtelomeric region driven by telomere attrition in any of the chromosomal arms identified in this study could alter gene expression thereby contributing to the "biological remembering" of PNS.

The quantification of (MN) frequencies in lymphocytes of patients with cancer has been used extensively as a biomarker of chromosomal damage and genomic instability. Being part of a larger study, we had access to MN frequencies scored in lymphocytes obtained from our study cohort at the five time points, as indicated earlier. Given that telomeric attrition has been associated with an increased frequency of chromosomal stability, we expected to see a negative correlation between telomere length and micronuclei frequency (shorter telomeres leading to higher MN levels). While negative relationships were observed between these variables, the

correlations did not reach significance at the five time points tested. It is quite feasible that while aberrations related to telomeric attrition following chemotherapy may arise, the majority of chromosomal changes being induced by chemotherapy may occur at sites other than the telomere, thereby reducing the association between telomere length and MN frequency.

Another variable lacking a significant predictive association with telomere length in our study cohort is the tumor characteristics. Shorter telomeres in **tumor** tissue have been associated with more aggressive subtypes, including luminal B, HER2 positive and triple negative tumors (Heaphy et al., 2011), but little is known about the association of a patient's **leukocyte** telomere length based on her breast tumor molecular sub-group characterization. In a recent study, Barczak, et al (2016) reported shorter telomere lengths in leukocytes derived from patients with higher stage breast cancer compared to patients at an early stage of the disease, but found no association between the histological cancer grade and leukocyte telomere length in patients with early or advanced stage breast cancer. In this study, we also did not find tumor stage, or tumor molecular sub-groups, to be predictive of lymphocyte telomere length. However, it is reasonable to conjecture this lack of association between telomere length and tumor characteristics could be due to the inclusion of only women with early stage breast cancer. Alternatively, leukocyte telomere length may not be predictive of tumor telomere lengths.

The most prominent strength of our study is its longitudinal design that allowed for the assessment of telomere length prior to chemotherapy, during active treatments and into survivorship. Moreover, most studies on telomere dynamics are limited by focusing on measuring average genomic telomere length using qPCR-based methodology. Our use of the chromosome-specific assay enabled us to assess individual telomeres. However, this later assay

is very labor intensive and expensive therefore, our assessment of telomere length using this method was limited to only baseline and mid-chemo, which in turn limited our comparisons to only two time points. Also, since high quality metaphases for the chromosome-specific assay, could not be obtained from a subset of our study participants, the sample size was decreased for this assay. Another limitation of this study was that our statistical analysis did not adjust the mixed effect models for all possible confounders. Therefore, our observations may have been affected by these confounders.

In summary, we showed that demographic factors significantly associated with telomere shortening were age and race. Using our chromosome-specific assay, we also showed that overall, telomere length in lymphocytes obtained from women treated for breast cancer is shortened following the initiation of chemotherapy treatment and that the change in telomere length (from baseline values) differs based on the type of chemotherapy used in treatment. We also demonstrated that this shortening doesn't affect all telomeres equally, with telomeres on only a subset of chromosomal arms being most impacted. Lastly, our study showed that pain was significantly related to telomere shortening in this study cohort.

Table 11: Chromosome-Specific Telomere Length and Cancer Risk

Reference	Study Sample	Method	Summary of Finding
Zheng et al., 2009	Cases: 153 women with breast cancer who have not been treated with chemotherapy or radiotherapy Controls: 159 age matched healthy women	Q-FISH	Short telomere on chromosome 9p is strongly associate with breast cancer risk
Zheng et al., 2011	Cases: 204 women with breast cancer who have not been treated with chemotherapy or radiotherapy Controls: 236 age matched healthy women	PNA-FISH	Short telomere length on chromosomes 9p,15p, 15q and Xp is associated with breast cancer risk in pre-menopausal women
Xing et al., 2009	Cases: 94 untreated patients with esophageal cancer Controls: 94 age, gender and ethnicity matched healthy subjects	STELA	Short telomere length on chromosomes 17p and 12q play a more prominent role in the etiology of esophageal cancer than 11q and 2p

Q-FISH= Quantitative Fluorescence In Situ Hybridization

PNA-FISH= Peptide Nucleic Acid Fluorescence In Situ Hybridization

STELA= Single Telomere Length Analysis

Table 12: Studies on the Effect of Doxorubicin (Adriamycin) on Telomere Biology

Reference	Sample type	Study outcome
Elmore et al., 2002	Mcf7 breast tumor cell line	Breast tumor cells treated with Adriamycin require both functional p53 and telomere dysfunction to exhibit a senescence phenotype
Mitchell et al., 2010	LiSa-2 ALT positive cell line derived from a poorly differentiated pleomorphic liposarcoma LS2 cell line derived from ALT-positive pleomorphic liposarcoma SW872 cell line expressing expressing both components of telomerase	Cell lines sensitivity to doxorubicin correlated to topoisomerase 2A (TOP2A) gene expression, with LiSa-2 cell line showing the highest sensitivity to doxorubicin and the highest expression of TOP2A followed by LS2 cell line SW872 cell line showed showed the lowest sensitivity to doxorubicin and the lowest expression of TOP2A
Buttiglieri et al., 2011	Mesenchymal stem cells derived from normal human bone marrow	Telomere loss following treatment with doxorubicin that was also associated with defects in proliferation and differentiation
Li et al., 2012	T-lymphocytes isolated from buffy coats of healthy individuals with stimulated telomerase activation Normal human lung fetal fibroblasts representing telomerase deficient cells	Rapid loss of telomeric DNA sequence, coupled with down-regulation of telomerase activity, repression of hTERT, TPP1 and POT1 (members of the shelterin complex) expression in T-lymphocytes and fibroblasts treated with doxorubicin resulting in telomere dysfunction in a high fraction of cells
Kato et al., 2013	Human cervical carcinoma cell line (HeLa), human normal fibroblast cell line (WI-38), human osteosarcoma cell lines (U-2-OS and Saos-2)	TRF1 and POT1 (members of the shelterin complex) mRNA levels were commonly down-regulated in both cancerous and normal cell lines following treatment with doxorubicin. On the other hand, RAP1 was up-regulated in a time-dependent manner in 2-OS cells only
Sanoff et al., 2014	33 women with early stage breast cancer (I-III) who were receiving doxorubicin, cychlophosmaide and taxane	Telomere length was measured before chemotherapy and 12 months after chemotherapy, with no significant change observed

Chapter 6

Conclusion and Future Directions

Concluding Remarks

Telomere biology plays a critical role in regulating cellular life span, aging and genomic instability. Therefore, telomere length has emerged as a prominent molecular marker of adverse health outcomes, such as breast cancer, which is among the most prevalent diseases worldwide but also one that has a high survival rate because of continuous advancements in detection and treatment strategies. Telomere length alterations in patients with breast cancer have been evaluated extensively as a diagnostic marker in breast tissue. However, research involving measuring telomere length in peripheral blood lymphocytes have focused primarily on investigating the potential link between telomere length and the risk of developing breast cancer. Clearly, there is a lack in the literature for studies investigating the relationship between genetic changes, particularly telomere attrition and the development and persistence of PNS under the influence of chemotherapy in patients with breast cancer.

Future Directions

This work was designed to assess telomere length in women with early stage breast cancer who were treated with chemotherapy, with the goal of improving our understanding of the role biological events play in the development and persistence of PNS. We have successfully

identified significant shortening in telomere length following the administration of chemotherapy in specific chromosomal arms. Furthermore, we have identified that patients in our study cohort having shorter telomere length on particular chromosomal arms reported higher pain intensity. Mammalian telomeric regions are enriched in signatures of heterochromatin including hypermethylation (Benetti, Garcia-Cao & Blasco, 2007). The heterochromatic signature of the telomeric region induces a position effect by spreading into the subtelomeric region as a function of length (Robin et al., 2014). We therefore, suspect that chromosomes showing significant telomere shortening may have an altered methylation profile in the subtelomeric region. This may in turn change the expression of genes associated with PNS if present. As mentioned earlier, this study is part of a larger study in which genome-wide methylation patterns were assessed using the 450K HumanMethylation Chip at baseline and mid-chemo time points. The methylation data could be used to determine if the distribution of sites showing methylation alterations from baseline to mid-chemo are more heavily localized to the subtelomeric regions of the telomeres that were showing significant shortening or the telomeres associated with pain. A change in the cell's methylation pattern(s) could have changed the expression of genes associated with pain, thereby allowing the biological remembering of this side effect into survivorship. Thus, we have already initiated additional studies to evaluate the possible presence of a relationship between methylation patterns and telomere length in this study cohort.

Interestingly, although not significant, a subset of our participants showed both shortening and lengthening of telomeres. This caught our attention as to what mechanism was responsible for this observation? The absence or insufficient amount of telomerase in adult somatic cells, points toward ALT as a plausible mechanism for increases in telomere lengths in our cohort. Support for this hypothesis is derived from the proposed effect of chemotherapeutic drugs on

different members of the shelterin complex which in turn could facilitate ALT through HR mediated DNA repair. To further investigate this hypothesis, we intend to determine ALT status by checking for the presence of telomere-sister chromatid exchange (T-SCE), which can be detected using chromosome-orientation Fluorescence In Situ Hybridization (CO-FISH) on metaphase spreads obtained from the study participants. This technique involves culturing cells for a single replication cycle with the incorporation of the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) for it's incorporation into the newly synthesized DNA strand and subsequent destruction through nuclease digestion. This leaves the template strand intact for FISH with telomere strand probes that can indicate the occurrence of a recombination event between sister chromatids (Bailey et al., 2001, Vera et al., 2008). Furthermore, the presence of telomere lengthening shown in our results could be also be addressed by determining the existence of associations between telomere length and different healthy life style behaviors (such as consuming a healthy diet, regular physical activity, practicing meditation), which have been associated with longer telomere lengths in several studies. Careful analysis of these variable that were obtained from our study cohort could aid in teasing apart the telomere lengthening observation.

The influence of inflammatory markers and oxidative stress on telomere length and their close association with PNS could provide another direction for expanding upon this study. Increased cell turnover and replicative senescence negatively affect telomere length. Gene expression levels of a classical marker of cellular senescence, CDKN2A (also known as p16), IL-6 and IL-8, as well as measures of a wide range of proinflammatory cytokines, including IL-6, were obtained from our study cohort. Therefore, our future studies will involve investigating the potential correlation between these variables and telomere length would provide a plausible

explanation for the observed telomere shortening in lymphocytes of patients with early stage breast cancer.

To the best of our knowledge, this is the first study to provide insights into the effect of chemotherapy on telomere length and their relationship with adverse side effects experienced in patients with breast cancer over a period of 2 years. If confirmed and expanded upon in future studies, these observations would be important for the recognition of lymphocyte telomere length as a biomarker for the identification of women who are at an increased risk of developing PNS and potentially designing therapeutic and intervention strategies that will be most effective in alleviating the adverse side effects in BC survivors and for ultimately improving their quality of life.

References Cited

References Cited

Abdallah P, Luciano P, Runge KW, Lisby M, Geli V, Gilson E, Teixeira MT. 2009. A two-step model for senescence triggered by a single critically short telomere. Nat Cell Biol 11(8):988-93.

Aboalela N, Lyon D, Elswick RK,Jr, Kelly DL, Brumelle J, Bear HD, Jackson-Cook C. 2015. Perceived stress levels, chemotherapy, radiation treatment and tumor characteristics are associated with a persistent increased frequency of somatic chromosomal instability in women diagnosed with breast cancer: A one year longitudinal study. PLoS One 10(7):e0133380.

Abut YC, Kitapcioglu D, Erkalp K, Toprak N, Boztepe A, Sivrikaya U, Paksoy I, Gur EK, Eren G, Bilen A. 2012. Job burnout in 159 anesthesiology trainees. Saudi J Anaesth 6(1):46-51.

Andrew T, Aviv A, Falchi M, Surdulescu GL, Gardner JP, Lu X, Kimura M, Kato BS, Valdes AM, Spector TD. 2006. Mapping genetic loci that determine leukocyte telomere length in a large sample of unselected female sibling pairs. Am J Hum Genet 78(3):480-6.

Aubert G and Lansdorp PM. 2008. Telomeres and aging. Physiol Rev 88(2):557-79.

Aubert G, Hills M, Lansdorp PM. 2012. Telomere length measurement-caveats and a critical assessment of the available technologies and tools. Mutat Res 730(1-2):59-67.

Aviv A. 2004. Telomeres and human aging: Facts and fibs. Sci Aging Knowledge Environ 2004(51):pe43.

Babizhayev MA, Savel'yeva EL, Moskvina SN, Yegorov YE. 2011. Telomere length is a biomarker of cumulative oxidative stress, biologic age, and an independent predictor of survival and therapeutic treatment requirement associated with smoking behavior. Am J Ther 18(6):e209-26.

Badger T, Segrin C, Dorros SM, Meek P, Lopez AM. 2007. Depression and anxiety in women with breast cancer and their partners. Nurs Res 56(1):44-53.

Bailey SM and Murnane JP. 2006. Telomeres, chromosome instability and cancer. Nucleic Acids Res 34(8):2408-17.

Bailey SM, Cornforth MN, Kurimasa A, Chen DJ, Goodwin EH. 2001. Strand-specific post replicative processing of mammalian telomeres. Science 293(5539):2462-5.

Baird DM, Rowson J, Wynford-Thomas D, Kipling D. 2003. Extensive allelic variation and ultrashort telomeres in senescent human cells. Nat Genet 33(2):203-7.

Barczak W, Rozwadowska N, Romaniuk A, Lipinska N, Lisiak N, Grodecka-Gazdecka S, Ksiazek K, Rubis B. 2016. Telomere length assessment in leukocytes presents potential diagnostic value in patients with breast cancer. Oncol Lett 11(3):2305-9.

Bardwell WA and Ancoli-Israel S. 2008. Breast cancer and fatigue. Sleep Med Clin 3(1):61-71.

Barrett JH, Iles MM, Dunning AM, Pooley KA. 2015. Telomere length and common disease: Study design and analytical challenges. Hum Genet 134(7):679-89.

Barwell J, Pangon L, Georgiou A, Docherty Z, Kesterton I, Ball J, Camplejohn R, Berg J, Aviv A, Gardner J, and others. 2007. Is telomere length in peripheral blood lymphocytes correlated with cancer susceptibility or radiosensitivity? Br J Cancer 97(12):1696-700.

Baur JA, Zou Y, Shay JW, Wright WE. 2001. Telomere position effect in human cells. Science 292(5524):2075-7.

Benetti R, Garcia-Cao M, Blasco MA. 2007. Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. Nat Genet 39(2):243-50.

Blackburn EH, Epel ES, Lin J. 2015. Human telomere biology: A contributory and interactive factor in aging, disease risks, and protection. Science 350(6265):1193-8.

Blasco MA. 2007. The epigenetic regulation of mammalian telomeres. Nat Rev Genet 8(4):299-309.

Bower JE. 2008. Behavioral symptoms in patients with breast cancer and survivors. J Clin Oncol 26(5):768-77.

Buttiglieri S, Ruella M, Risso A, Spatola T, Silengo L, Avvedimento EV, Tarella C. 2011. The aging effect of chemotherapy on cultured human mesenchymal stem cells. Exp Hematol 39(12):1171-81.

Campisi J and d'Adda di Fagagna F. 2007. Cellular senescence: When bad things happen to good cells. Nat Rev Mol Cell Biol 8(9):729-40.

Caraceni A. 2001. Evaluation and assessment of cancer pain and cancer pain treatment. Acta Anaesthesiol Scand 45(9):1067-75.

Carlson LE, Beattie TL, Giese-Davis J, Faris P, Tamagawa R, Fick LJ, Degelman ES, Speca M. 2015. Mindfulness-based cancer recovery and supportive-expressive therapy maintain telomere length relative to controls in distressed breast cancer survivors. Cancer 121(3):476-84.

Cassidy A, De Vivo I, Liu Y, Han J, Prescott J, Hunter DJ, Rimm EB. 2010. Associations between diet, lifestyle factors, and telomere length in women. Am J Clin Nutr 91(5):1273-80.

Cawthon RM. 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. Nucleic Acids Res 37(3):e21.

Cawthon RM. 2002. Telomere measurement by quantitative PCR. Nucleic Acids Res 30(10):e47.

Cesare AJ and Reddel RR. 2008. Telomere uncapping and alternative lengthening of telomeres. Mech Ageing Dev 129(1-2):99-108.

Cherkas LF, Hunkin JL, Kato BS, Richards JB, Gardner JP, Surdulescu GL, Kimura M, Lu X, Spector TD, Aviv A. 2008. The association between physical activity in leisure time and leukocyte telomere length. Arch Intern Med 168(2):154-8.

Cleeland CS, Bennett GJ, Dantzer R, Dougherty PM, Dunn AJ, Meyers CA, Miller AH, Payne R, Reuben JM, Wang XS, and others. 2003. Are the symptoms of cancer and cancer treatment due to a shared biologic mechanism? A cytokine-immunologic model of cancer symptoms. Cancer 97(11):2919-25.

Cohen S, Kamarck T, Mermelstein R. 1983. A global measure of perceived stress. J Health Soc Behav 24(4):385-96.

Collado M, Blasco MA, Serrano M. 2007. Cellular senescence in cancer and aging. Cell 130(2):223-33.

Conomos D, Pickett HA, Reddel RR. 2013. Alternative lengthening of telomeres: Remodeling the telomere architecture. Front Oncol 3:27.

d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP. 2003. A DNA damage checkpoint response in telomere-initiated senescence. Nature 426(6963):194-8.

Damjanovic AK, Yang Y, Glaser R, Kiecolt-Glaser JK, Nguyen H, Laskowski B, Zou Y, Beversdorf DQ, Weng NP. 2007. Accelerated telomere erosion is associated with a declining immune function of caregivers of alzheimer's disease patients. J Immunol 179(6):4249-54.

Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. 2008. From inflammation to sickness and depression: When the immune system subjugates the brain. Nat Rev Neurosci 9(1):46-56.

de Beer D, Volzmann J, Kalka C, Baerlocher GM. 2015. Longitudinal telomere erosion in lymphocyte subsets of patients with atherosclerotic peripheral arterial disease (PAD). J Clin Diagn Res 9(3):OM01-3.

de Lange T. 2009. How telomeres solve the end-protection problem. Science 326(5955):948-52.

de Lange T. 2002. Protection of mammalian telomeres. Oncogene 21(4):532-40.

De Vivo I, Prescott J, Wong JY, Kraft P, Hankinson SE, Hunter DJ. 2009. A prospective study of relative telomere length and postmenopausal breast cancer risk. Cancer Epidemiol Biomarkers Prev 18(4):1152-6.

Denchi EL and de Lange T. 2007. Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature 448(7157):1068-71.

DeVon HA, Piano MR, Rosenfeld AG, Hoppensteadt DA. 2014. The association of pain with protein inflammatory biomarkers: A review of the literature. Nurs Res 63(1):51-62.

D'Mello MJ, Ross SA, Anand SS, Gerstein H, McQueen M, Yusuf S, Pare G. 2016. Telomere length and risk of myocardial infarction in a MultiEthnic population: The INTERHEART study. J Am Coll Cardiol 67(15):1863-5.

Dodd MJ, Cho MH, Cooper BA, Miaskowski C. 2010. The effect of symptom clusters on functional status and quality of life in women with breast cancer. Eur J Oncol Nurs 14(2):101-10.

Drury SS, Esteves K, Hatch V, Woodbury M, Borne S, Adamski A, Theall KP. 2015. Setting the trajectory: Racial disparities in newborn telomere length. J Pediatr 166(5):1181-6.

Dunham MA, Neumann AA, Fasching CL, Reddel RR. 2000. Telomere maintenance by recombination in human cells. Nat Genet 26(4):447-50.

Effros RB. 2009. Kleemeier award lecture 2008--the canary in the coal mine: Telomeres and human healthspan. J Gerontol A Biol Sci Med Sci 64(5):511-5.

Egholm M, Buchardt O, Christensen L, Behrens C, Freier SM, Driver DA, Berg RH, Kim SK, Norden B, Nielsen PE. 1993. PNA hybridizes to complementary oligonucleotides obeying the watson-crick hydrogen-bonding rules. Nature 365(6446):566-8.

Elmore LW, Rehder CW, Di X, McChesney PA, Jackson-Cook CK, Gewirtz DA, Holt SE. 2002. Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction. J Biol Chem 277(38):35509-15.

Epel ES, Blackburn EH, Lin J, Dhabhar FS, Adler NE, Morrow JD, Cawthon RM. 2004. Accelerated telomere shortening in response to life stress. Proc Natl Acad Sci U S A 101(49):17312-5.

Farzaneh-Far R, Lin J, Epel ES, Harris WS, Blackburn EH, Whooley MA. 2010. Association of marine omega-3 fatty acid levels with telomeric aging in patients with coronary heart disease. Jama 303(3):250-7.

Fauzee NJ. 2011. Taxanes: Promising anti-cancer drugs. Asian Pac J Cancer Prev 12(4):837-51.

Ferreira VT, Dibai-Filho AV, Kelly de Oliveira A, Gomes CA, Melo ES, Maria de Almeida A. 2015. Assessing the impact of pain on the life of breast cancer survivors using the brief pain inventory. J Phys Ther Sci 27(5):1361-3.

Fumagalli M, Rossiello F, Clerici M, Barozzi S, Cittaro D, Kaplunov JM, Bucci G, Dobreva M, Matti V, Beausejour CM, and others. 2012. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nat Cell Biol 14(4):355-65.

Gewirtz DA. 1999. A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin. Biochem Pharmacol 57(7):727-41.

Gilson E and Londono-Vallejo A. 2007. Telomere length profiles in humans: All ends are not equal. Cell Cycle 6(20):2486-94.

Giordano A, Tagliabue E, Pupa SM. 2012. Promise and failure of targeted therapy in breast cancer. Front Biosci (Schol Ed) 4:356-74.

Global Burden of Disease Cancer Collaboration, Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, MacIntyre MF, Allen C, Hansen G, Woodbrook R, and others. 2015. The global burden of cancer 2013. JAMA Oncol 1(4):505-27.

Gocha AR, Harris J, Groden J. 2013. Alternative mechanisms of telomere lengthening: Permissive mutations, DNA repair proteins and tumorigenic progression. Mutat Res 743-744:142-50.

Gramatges MM, Telli ML, Balise R, Ford JM. 2010. Longer relative telomere length in blood from women with sporadic and familial breast cancer compared with healthy controls. Cancer Epidemiol Biomarkers Prev 19(2):605-13.

Gualtieri CT and Johnson LG. 2006. Reliability and validity of a computerized neurocognitive test battery, CNS vital signs. Arch Clin Neuropsychol 21(7):623-43.

Guan JZ, Guan WP, Maeda T, Makino N. 2013. Analysis of telomere length and subtelomeric methylation of circulating leukocytes in women with alzheimer's disease. Aging Clin Exp Res 25(1):17-23.

Guo X, Deng Y, Lin Y, Cosme-Blanco W, Chan S, He H, Yuan G, Brown EJ, Chang S. 2007. Dysfunctional telomeres activate an ATM-ATR-dependent DNA damage response to suppress tumorigenesis. Embo j 26(22):4709-19.

Hayashi MT, Cesare AJ, Fitzpatrick JA, Lazzerini-Denchi E, Karlseder J. 2012. A telomere-dependent DNA damage checkpoint induced by prolonged mitotic arrest. Nat Struct Mol Biol 19(4):387-94.

Heaphy CM and Meeker AK. 2011. The potential utility of telomere-related markers for cancer diagnosis. J Cell Mol Med 15(6):1227-38.

Heaphy CM, Subhawong AP, Gross AL, Konishi Y, Kouprina N, Argani P, Visvanathan K, Meeker AK. 2011. Shorter telomeres in luminal B, HER-2 and triple-negative breast cancer subtypes. Mod Pathol 24(2):194-200.

Hemann MT, Strong MA, Hao LY, Greider CW. 2001. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. Cell 107(1):67-77.

Hofree M, Shen JP, Carter H, Gross A, Ideker T. 2013. Network-based stratification of tumor mutations. Nat Methods 10(11):1108-15.

Hoxha M, Dioni L, Bonzini M, Pesatori AC, Fustinoni S, Cavallo D, Carugno M, Albetti B, Marinelli B, Schwartz J, and others. 2009. Association between leukocyte telomere shortening and exposure to traffic pollution: A cross-sectional study on traffic officers and indoor office workers. Environ Health 8:41,069X-8-41.

Huang HY, Helzlsouer KJ, Appel LJ. 2000. The effects of vitamin C and vitamin E on oxidative DNA damage: Results from a randomized controlled trial. Cancer Epidemiol Biomarkers Prev 9(7):647-52.

Hunt SC, Chen W, Gardner JP, Kimura M, Srinivasan SR, Eckfeldt JH, Berenson GS, Aviv A. 2008. Leukocyte telomeres are longer in african americans than in whites: The national heart, lung, and blood institute family heart study and the bogalusa heart study. Aging Cell 7(4):451-8.

Hurria A, Somlo G, Ahles T. 2007. Renaming "chemobrain". Cancer Invest 25(6):373-7.

Innes KE, Selfe TK, Brown CJ, Rose KM, Thompson-Heisterman A. 2012. The effects of meditation on perceived stress and related indices of psychological status and sympathetic activation in persons with alzheimer's disease and their caregivers: A pilot study. Evid Based Complement Alternat Med 2012:927509.

Irie M, Asami S, Ikeda M, Kasai H. 2003. Depressive state relates to female oxidative DNA damage via neutrophil activation. Biochem Biophys Res Commun 311(4):1014-8.

Jacobs PA and Strong JA. 1959. A case of human intersexuality having a possible XXY sex-determining mechanism. Nature 183(4657):302-3.

Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ. 2000. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. Cancer Res 60(1):184-90.

Janssen A and Medema RH. 2011. Mitosis as an anti-cancer target. Oncogene 30(25):2799-809.

Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. 2009. Cancer statistics, 2009. CA Cancer J Clin 59(4):225-49.

Jemal A, Simard EP, Dorell C, Noone AM, Markowitz LE, Kohler B, Eheman C, Saraiya M, Bandi P, Saslow D, and others. 2013. Annual report to the nation on the status of cancer, 1975-2009, featuring the burden and trends in human papillomavirus(HPV)-associated cancers and HPV vaccination coverage levels. J Natl Cancer Inst 105(3):175-201.

Jiao J, Kang JX, Tan R, Wang J, Zhang Y. 2012. Multiplex time-reducing quantitative polymerase chain reaction assay for determination of telomere length in blood and tissue DNA. Anal Bioanal Chem 403(1):157-66.

Jung MS, Zhang M, Askren MK, Berman MG, Peltier S, Hayes DF, Therrien B, Reuter-Lorenz PA, Cimprich B. 2016. Cognitive dysfunction and symptom burden in women treated for breast cancer: A prospective behavioral and fMRI analysis. Brain Imaging Behav .

Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. 1999. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science 283(5406):1321-5.

Kato M, Nakayama M, Agata M, Yoshida K. 2013. Gene expression levels of human shelterin complex and shelterin-associated factors regulated by the topoisomerase II inhibitors doxorubicin and etoposide in human cultured cells. Tumour Biol 34(2):723-33.

Kawanishi S, Hiraku Y, Oikawa S. 2001. Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging. Mutat Res 488(1):65-76.

Kim HJ, Barsevick AM, Fang CY, Miaskowski C. 2012. Common biological pathways underlying the psychoneurological symptom cluster in cancer patients. Cancer Nurs 35(6):E1-E20.

Kim S, Sandler DP, Carswell G, De Roo LA, Parks CG, Cawthon R, Weinberg CR, Taylor JA. 2011. Telomere length in peripheral blood and breast cancer risk in a prospective case-cohort analysis: Results from the sister study. Cancer Causes Control 22(7):1061-6.

Kregel KC and Zhang HJ. 2007. An integrated view of oxidative stress in aging: Basic mechanisms, functional effects, and pathological considerations. Am J Physiol Regul Integr Comp Physiol 292(1):R18-36.

Krejci K and Koch J. 1998. Improved detection and comparative sizing of human chromosomal telomeres in situ. Chromosoma 107(3):198-203.

Lansdorp PM, Verwoerd NP, van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK, Tanke HJ. 1996. Heterogeneity in telomere length of human chromosomes. Hum Mol Genet 5(5):685-91.

Leach NT and Jackson-Cook C. 2001. The application of spectral karyotyping (SKY) and fluorescent in situ hybridization (FISH) technology to determine the chromosomal content(s) of micronuclei. Mutat Res 495(1-2):11-9.

Leach NT, Rehder C, Jensen K, Holt S, Jackson-Cook C. 2004. Human chromosomes with shorter telomeres and large heterochromatin regions have a higher frequency of acquired somatic cell aneuploidy. Mech Ageing Dev 125(8):563-73.

Lee K, Cho M, Miaskowski C, Dodd M. 2004. Impaired sleep and rhythms in persons with cancer. Sleep Med Rev 8(3):199-212.

Li P, Hou M, Lou F, Bjorkholm M, Xu D. 2012. Telomere dysfunction induced by chemotherapeutic agents and radiation in normal human cells. Int J Biochem Cell Biol 44(9):1531-40.

Lindqvist D, Epel ES, Mellon SH, Penninx BW, Revesz D, Verhoeven JE, Reus VI, Lin J, Mahan L, Hough CM, and others. 2015. Psychiatric disorders and leukocyte telomere length: Underlying mechanisms linking mental illness with cellular aging. Neurosci Biobehav Rev 55:333-64.

Liu M, Hales BF, Robaire B. 2014. Effects of four chemotherapeutic agents, bleomycin, etoposide, cisplatin, and cyclophosphamide, on DNA damage and telomeres in a mouse spermatogonial cell line. Biol Reprod 90(4):72.

Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. 2013. The hallmarks of aging. Cell 153(6):1194-217.

Louati K and Berenbaum F. 2015. Fatigue in chronic inflammation - a link to pain pathways. Arthritis Res Ther 17:254,015-0784-1.

Lu Y, Leong W, Guerin O, Gilson E, Ye J. 2013. Telomeric impact of conventional chemotherapy. Front Med 7(4):411-7.

Lynch SM, Peek MK, Mitra N, Ravichandran K, Branas C, Spangler E, Zhou W, Paskett ED, Gehlert S, DeGraffinreid C, and others. 2016. Race, ethnicity, psychosocial factors, and telomere length in a multicenter setting. PLoS One 11(1):e0146723.

Lyon D, Elmore L, Aboalela N, Merrill-Schools J, McCain N, Starkweather A, Elswick RK,Jr, Jackson-Cook C. 2014. Potential epigenetic mechanism(s) associated with the persistence of psychoneurological symptoms in women receiving chemotherapy for breast cancer: A hypothesis. Biol Res Nurs 16(2):160-74.

Maccormick RE. 2006. Possible acceleration of aging by adjuvant chemotherapy: A cause of early onset frailty? Med Hypotheses 67(2):212-5.

Makarov VL, Hirose Y, Langmore JP. 1997. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell 88(5):657-66.

Malhotra GK, Zhao X, Band H, Band V. 2010. Histological, molecular and functional subtypes of breast cancers. Cancer Biol Ther 10(10):955-60.

Meeker AK, Hicks JL, Gabrielson E, Strauss WM, De Marzo AM, Argani P. 2004. Telomere shortening occurs in subsets of normal breast epithelium as well as in situ and invasive carcinoma. Am J Pathol 164(3):925-35.

Megarbane A, Ravel A, Mircher C, Sturtz F, Grattau Y, Rethore MO, Delabar JM, Mobley WC. 2009. The 50th anniversary of the discovery of trisomy 21: The past, present, and future of research and treatment of down syndrome. Genet Med 11(9):611-6.

Mendoza TR, Wang XS, Cleeland CS, Morrissey M, Johnson BA, Wendt JK, Huber SL. 1999. The rapid assessment of fatigue severity in cancer patients: Use of the brief fatigue inventory. Cancer 85(5):1186-96.

Meyers CA, Albitar M, Estey E. 2005. Cognitive impairment, fatigue, and cytokine levels in patients with acute myelogenous leukemia or myelodysplastic syndrome. Cancer 104(4):788-93.

Miaskowski C and Aouizerat BE. 2007. Is there a biological basis for the clustering of symptoms? Semin Oncol Nurs 23(2):99-105.

Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. 2004. Anthracyclines: Molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 56(2):185-229.

Mirjolet C, Boidot R, Saliques S, Ghiringhelli F, Maingon P, Crehange G. 2015. The role of telomeres in predicting individual radiosensitivity of patients with cancer in the era of personalized radiotherapy. Cancer Treat Rev 41(4):354-60.

Mitchell MA, Johnson JE, Pascarelli K, Beeharry N, Chiourea M, Gagos S, Lev D, von Mehren M, Kipling D, Broccoli D. 2010. Doxorubicin resistance in a novel in vitro model of human pleomorphic liposarcoma associated with alternative lengthening of telomeres. Mol Cancer Ther 9(3):682-92.

Montpetit AJ, Alhareeri AA, Montpetit M, Starkweather AR, Elmore LW, Filler K, Mohanraj L, Burton CW, Menzies VS, Lyon DE, and others. 2014. Telomere length: A review of methods for measurement. Nurs Res 63(4):289-99.

Moyzis RK, Buckingham JM, Cram LS, Dani M, Deaven LL, Jones MD, Meyne J, Ratliff RL, Wu JR. 1988. A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. Proc Natl Acad Sci U S A 85(18):6622-6.

Muezzinler A, Zaineddin AK, Brenner H. 2013. A systematic review of leukocyte telomere length and age in adults. Ageing Res Rev 12(2):509-19.

Muezzinler A, Mons U, Dieffenbach AK, Butterbach K, Saum KU, Schick M, Stammer H, Boukamp P, Holleczek B, Stegmaier C, and others. 2016. Body mass index and leukocyte telomere length dynamics among older adults: Results from the ESTHER cohort. Exp Gerontol 74:1-8.

Muezzinler A, Mons U, Dieffenbach AK, Butterbach K, Saum KU, Schick M, Stammer H, Boukamp P, Holleczek B, Stegmaier C, and others. 2015. Smoking habits and leukocyte telomere length dynamics among older adults: Results from the ESTHER cohort. Exp Gerontol 70:18-25.

Myers MB. 2016. Targeted therapies with companion diagnostics in the management of breast cancer: Current perspectives. Pharmgenomics Pers Med 9:7-16.

Nawrot TS, Staessen JA, Gardner JP, Aviv A. 2004. Telomere length and possible link to X chromosome. Lancet 363(9408):507-10.

Njajou OT, Cawthon RM, Damcott CM, Wu SH, Ott S, Garant MJ, Blackburn EH, Mitchell BD, Shuldiner AR, Hsueh WC. 2007. Telomere length is paternally inherited and is associated with parental lifespan. Proc Natl Acad Sci U S A 104(29):12135-9.

Nordfjall K, Larefalk A, Lindgren P, Holmberg D, Roos G. 2005. Telomere length and heredity: Indications of paternal inheritance. Proc Natl Acad Sci U S A 102(45):16374-8.

Oeseburg H, de Boer RA, van Gilst WH, van der Harst P. 2010. Telomere biology in healthy aging and disease. Pflugers Arch 459(2):259-68.

Okuda K, Bardeguez A, Gardner JP, Rodriguez P, Ganesh V, Kimura M, Skurnick J, Awad G, Aviv A. 2002. Telomere length in the newborn. Pediatr Res 52(3):377-81.

Palm W and de Lange T. 2008. How shelterin protects mammalian telomeres. Annu Rev Genet 42:301-34.

Parker WB. 2009. Enzymology of purine and pyrimidine antimetabolites used in the treatment of cancer. Chem Rev 109(7):2880-93.

Patel R, McIntosh L, McLaughlin J, Brooke S, Nimon V, Sapolsky R. 2002. Disruptive effects of glucocorticoids on glutathione peroxidase biochemistry in hippocampal cultures. J Neurochem 82(1):118-25.

Pavanello S, Pesatori AC, Dioni L, Hoxha M, Bollati V, Siwinska E, Mielzynska D, Bolognesi C, Bertazzi PA, Baccarelli A. 2010. Shorter telomere length in peripheral blood lymphocytes of workers exposed to polycyclic aromatic hydrocarbons. Carcinogenesis 31(2):216-21.

Pawelczyk T, Szymanska B, Grancow-Grabka M, Kotlicka-Antczak M, Pawelczyk A. 2015. Telomere length in blood cells is related to the chronicity, severity, and recurrence rate of schizophrenia. Neuropsychiatr Dis Treat 11:1493-503.

Pellatt AJ, Wolff RK, Torres-Mejia G, John EM, Herrick JS, Lundgreen A, Baumgartner KB, Giuliano AR, Hines LM, Fejerman L, and others. 2013. Telomere length, telomere-related genes, and breast cancer risk: The breast cancer health disparities study. Genes Chromosomes Cancer 52(7):595-609.

Prather AA, Gurfein B, Moran P, Daubenmier J, Acree M, Bacchetti P, Sinclair E, Lin J, Blackburn E, Hecht FM, and others. 2015. Tired telomeres: Poor global sleep quality, perceived stress, and telomere length in immune cell subsets in obese men and women. Brain Behav Immun 47:155-62.

Pusztai L, Mendoza TR, Reuben JM, Martinez MM, Willey JS, Lara J, Syed A, Fritsche HA, Bruera E, Booser D, and others. 2004. Changes in plasma levels of inflammatory cytokines in response to paclitaxel chemotherapy. Cytokine 25(3):94-102.

Qu S, Wen W, Shu XO, Chow WH, Xiang YB, Wu J, Ji BT, Rothman N, Yang G, Cai Q, and others. 2013. Association of leukocyte telomere length with breast cancer risk: Nested case-control findings from the shanghai women's health study. Am J Epidemiol 177(7):617-24.

Quiles JL, Huertas JR, Battino M, Mataix J, Ramirez-Tortosa MC. 2002. Antioxidant nutrients and adriamycin toxicity. Toxicology 180(1):79-95.

Rai R, Chen Y, Lei M, Chang S. 2016. TRF2-RAP1 is required to protect telomeres from engaging in homologous recombination-mediated deletions and fusions. Nat Commun 7:10881.

Robin JD, Ludlow AT, Batten K, Magdinier F, Stadler G, Wagner KR, Shay JW, Wright WE. 2014. Telomere position effect: Regulation of gene expression with progressive telomere shortening over long distances. Genes Dev 28(22):2464-76.

Sanoff HK, Deal AM, Krishnamurthy J, Torrice C, Dillon P, Sorrentino J, Ibrahim JG, Jolly TA, Williams G, Carey LA, and others. 2014. Effect of cytotoxic chemotherapy on markers of molecular age in patients with breast cancer. J Natl Cancer Inst 106(4):dju057.

Saretzki G and Von Zglinicki T. 2002. Replicative aging, telomeres, and oxidative stress. Ann N Y Acad Sci 959:24-9.

Sasaki A, Ishiuchi S, Kanda T, Hasegawa M, Nakazato Y. 2001. Analysis of interleukin-6 gene expression in primary human gliomas, glioblastoma xenografts, and glioblastoma cell lines. Brain Tumor Pathol 18(1):13-21.

Sassenroth D, Meyer A, Salewsky B, Kroh M, Norman K, Steinhagen-Thiessen E, Demuth I. 2015. Sports and exercise at different ages and leukocyte telomere length in later life--data from the berlin aging study II (BASE-II). PLoS One 10(12):e0142131.

Satija A, Ahmed SM, Gupta R, Ahmed A, Rana SP, Singh SP, Mishra S, Bhatnagar S. 2014. Breast cancer pain management - a review of current & novel therapies. Indian J Med Res 139(2):216-25.

Schroder CP, Wisman GB, de Jong S, van der Graaf WT, Ruiters MH, Mulder NH, de Leij LF, van der Zee AG, de Vries EG. 2001. Telomere length in breast cancer patients before and after chemotherapy with or without stem cell transplantation. Br J Cancer 84(10):1348-53.

Schutte NS and Malouff JM. 2014. A meta-analytic review of the effects of mindfulness meditation on telomerase activity. Psychoneuroendocrinology 42:45-8.

Senchenkov A, Litvak DA, Cabot MC. 2001. Targeting ceramide metabolism--a strategy for overcoming drug resistance. J Natl Cancer Inst 93(5):347-57.

Sfeir A and de Lange T. 2012. Removal of shelterin reveals the telomere end-protection problem. Science 336(6081):593-7.

Shalev I, Entringer S, Wadhwa PD, Wolkowitz OM, Puterman E, Lin J, Epel ES. 2013. Stress and telomere biology: A lifespan perspective. Psychoneuroendocrinology 38(9):1835-42.

Shay JW and Wright WE. 2005. Senescence and immortalization: Role of telomeres and telomerase. Carcinogenesis 26(5):867-74.

Shen J, Terry MB, Gurvich I, Liao Y, Senie RT, Santella RM. 2007. Short telomere length and breast cancer risk: A study in sister sets. Cancer Res 67(11):5538-44.

Shen J, Gammon MD, Terry MB, Wang Q, Bradshaw P, Teitelbaum SL, Neugut AI, Santella RM. 2009. Telomere length, oxidative damage, antioxidants and breast cancer risk. Int J Cancer 124(7):1637-43.

Shiels PG, McGlynn LM, MacIntyre A, Johnson PC, Batty GD, Burns H, Cavanagh J, Deans KA, Ford I, McConnachie A, and others. 2011. Accelerated telomere attrition is associated with relative household income, diet and inflammation in the pSoBid cohort. PLoS One 6(7):e22521.

Siegel RL, Miller KD, Jemal A. 2016. Cancer statistics, 2016. CA Cancer J Clin 66(1):7-30.

Slagboom PE, Droog S, Boomsma DI. 1994. Genetic determination of telomere size in humans: A twin study of three age groups. Am J Hum Genet 55(5):876-82.

Snaith RP. 2003. The hospital anxiety and depression scale. Health Qual Life Outcomes 1:29.

Soares-Miranda L, Imamura F, Siscovick D, Jenny NS, Fitzpatrick AL, Mozaffarian D. 2015. Physical activity, physical fitness, and leukocyte telomere length: The cardiovascular health study. Med Sci Sports Exerc 47(12):2525-34.

Soler D, Genesca A, Arnedo G, Egozcue J, Tusell L. 2005. Telomere dysfunction drives chromosomal instability in human mammary epithelial cells. Genes Chromosomes Cancer 44(4):339-50.

Song Z, von Figura G, Liu Y, Kraus JM, Torrice C, Dillon P, Rudolph-Watabe M, Ju Z, Kestler HA, Sanoff H, and others. 2010. Lifestyle impacts on the aging-associated expression of biomarkers of DNA damage and telomere dysfunction in human blood. Aging Cell 9(4):607-15.

Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, Deng S, Johnsen H, Pesich R, Geisler S, and others. 2003. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 100(14):8418-23.

Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, and others. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98(19):10869-74.

Southern EM. 1979. Measurement of DNA length by gel electrophoresis. Anal Biochem 100(2):319-23.

Stadler G, Rahimov F, King OD, Chen JC, Robin JD, Wagner KR, Shay JW, Emerson CP,Jr, Wright WE. 2013. Telomere position effect regulates DUX4 in human facioscapulohumeral muscular dystrophy. Nat Struct Mol Biol 20(6):671-8.

Starkweather AR, Lyon DE, Schubert CM. 2013. Pain and inflammation in women with early-stage breast cancer prior to induction of chemotherapy. Biol Res Nurs 15(2):234-41.

Starkweather AR, Alhaeeri AA, Montpetit A, Brumelle J, Filler K, Montpetit M, Mohanraj L, Lyon DE, Jackson-Cook CK. 2014. An integrative review of factors associated with telomere length and implications for biobehavioral research. Nurs Res 63(1):36-50.

Stingl J and Caldas C. 2007. Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Rev Cancer 7(10):791-9.

Svenson U, Nordfjall K, Stegmayr B, Manjer J, Nilsson P, Tavelin B, Henriksson R, Lenner P, Roos G. 2008. Breast cancer survival is associated with telomere length in peripheral blood cells. Cancer Res 68(10):3618-23.

Tempaku PF, Mazzotti DR, Tufik S. 2015. Telomere length as a marker of sleep loss and sleep disturbances: A potential link between sleep and cellular senescence. Sleep Med 16(5):559-63.

Thompson AM and Moulder-Thompson SL. 2012. Neoadjuvant treatment of breast cancer. Ann Oncol 23 Suppl 10:x231-6.

Thorn CF, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein TE, Altman RB. 2011. Doxorubicin pathways: Pharmacodynamics and adverse effects. Pharmacogenet Genomics 21(7):440-6.

Valdes AM, Andrew T, Gardner JP, Kimura M, Oelsner E, Cherkas LF, Aviv A, Spector TD. 2005. Obesity, cigarette smoking, and telomere length in women. Lancet 366(9486):662-4.

van Deutekom JC, Bakker E, Lemmers RJ, van der Wielen MJ, Bik E, Hofker MH, Padberg GW, Frants RR. 1996. Evidence for subtelomeric exchange of 3.3 kb tandemly repeated units between chromosomes 4q35 and 10q26: Implications for genetic counselling and etiology of FSHD1. Hum Mol Genet 5(12):1997-2003.

van Steensel B, Smogorzewska A, de Lange T. 1998. TRF2 protects human telomeres from end-to-end fusions. Cell 92(3):401-13.

Vasa-Nicotera M, Brouilette S, Mangino M, Thompson JR, Braund P, Clemitson JR, Mason A, Bodycote CL, Raleigh SM, Louis E, and others. 2005. Mapping of a major locus that determines telomere length in humans. Am J Hum Genet 76(1):147-51.

Vera E and Blasco MA. 2012. Beyond average: Potential for measurement of short telomeres. Aging (Albany NY) 4(6):379-92.

Vera E, Canela A, Fraga MF, Esteller M, Blasco MA. 2008. Epigenetic regulation of telomeres in human cancer. Oncogene 27(54):6817-33.

Viale G. 2012. The current state of breast cancer classification. Ann Oncol 23 Suppl 10:x207-10.

von Zglinicki T. 2002. Oxidative stress shortens telomeres. Trends Biochem Sci 27(7):339-44.

von Zglinicki T. 2000. Role of oxidative stress in telomere length regulation and replicative senescence. Ann N Y Acad Sci 908:99-110.

Wang YY, Chen AF, Wang HZ, Xie LY, Sui KX, Zhang QY. 2011. Association of shorter mean telomere length with large artery stiffness in patients with coronary heart disease. Aging Male 14(1):27-32.

Watters JL, Satia JA, Kupper LL. 2008. Correlates of antioxidant nutrients and oxidative DNA damage differ by race in a cross-sectional study of healthy african american and white adults. Nutr Res 28(9):565-76.

Watters JL, Satia JA, Kupper LL, Swenberg JA, Schroeder JC, Switzer BR. 2007. Associations of antioxidant nutrients and oxidative DNA damage in healthy african-american and white adults. Cancer Epidemiol Biomarkers Prev 16(7):1428-36.

Weinberger T, Forrester A, Markov D, Chism K, Kunkel EJ. 2010. Women at a dangerous intersection: Diagnosis and treatment of depression and related disorders in patients with breast cancer. Psychiatr Clin North Am 33(2):409-22.

Westbrook K and Stearns V. 2013. Pharmacogenomics of breast cancer therapy: An update. Pharmacol Ther 139(1):1-11.

Wright WE, Tesmer VM, Huffman KE, Levene SD, Shay JW. 1997. Normal human chromosomes have long G-rich telomeric overhangs at one end. Genes Dev 11(21):2801-9.

Xing J, Ajani JA, Chen M, Izzo J, Lin J, Chen Z, Gu J, Wu X. 2009. Constitutive short telomere length of chromosome 17p and 12q but not 11q and 2p is associated with an increased risk for esophageal cancer. Cancer Prev Res (Phila) 2(5):459-65.

Xu L, Li S, Stohr BA. 2013. The role of telomere biology in cancer. Annu Rev Pathol 8:49-78.

Xu Z, Duc KD, Holcman D, Teixeira MT. 2013. The length of the shortest telomere as the major determinant of the onset of replicative senescence. Genetics 194(4):847-57.

Yang F, Teves SS, Kemp CJ, Henikoff S. 2014. Doxorubicin, DNA torsion, and chromatin dynamics. Biochim Biophys Acta 1845(1):84-9.

Zanni GR and Wick JY. 2011. Telomeres: Unlocking the mystery of cell division and aging. Consult Pharm 26(2):78-90.

Zhang J, Rane G, Dai X, Shanmugam MK, Arfuso F, Samy RP, Lai MK, Kappei D, Kumar AP, Sethi G. 2016. Ageing and the telomere connection: An intimate relationship with inflammation. Ageing Res Rev 25:55-69.

Zheng YL, Loffredo CA, Shields PG, Selim SM. 2009. Chromosome 9 arm-specific telomere length and breast cancer risk. Carcinogenesis 30(8):1380-6.

Zheng YL, Zhou X, Loffredo CA, Shields PG, Sun B. 2011. Telomere deficiencies on chromosomes 9p, 15p, 15q and xp: Potential biomarkers for breast cancer risk. Hum Mol Genet 20(2):378-86.

Zheng YL, Hu N, Sun Q, Wang C, Taylor PR. 2009. Telomere attrition in cancer cells and telomere length in tumor stroma cells predict chromosome instability in esophageal squamous cell carcinoma: A genome-wide analysis. Cancer Res 69(4):1604-14.

Zheng YL, Ambrosone C, Byrne C, Davis W, Nesline M, McCann SE. 2010. Telomere length in blood cells and breast cancer risk: Investigations in two case-control studies. Breast Cancer Res Treat 120(3):769-75.

Zhu X, Han W, Xue W, Zou Y, Xie C, Du J, Jin G. 2016. The association between telomere length and cancer risk in population studies. Sci Rep 6:22243.

Vita

Areej Abdulaziz Alhareeri was born on April 8, 1983, in Riyadh, Saudi Arabia. She graduated from Dar Assalam high school, Riyadh, Saudi Arabia in 2000. She received her Bachelor of Science in Clinical Laboratory Sciences, from King Saud University, Riyadh, Saudi Arabia in 2005 and subsequently worked as a medical technologist in a clinical cytogenetics laboratory in king Abdulaziz Medical City, Riyadh, Saudi Arabia for four years. In 2009, she obtained the American Society of Clinical Pathology (ASCP) Board of Certification in Cytogenetics. In the same year, Areej received a scholarship from King Saud University for Health Sciences to pursue her graduate study in the field of genetics. She joined the Department of Human and Molecular Genetics in Virginia Commonwealth University in 2010 and earned her Master's of Science degree in Human Genetics in 2013.